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The Indirect Effect of BVDV on Immune Suppression: The Role of Infected Macrophages in Lymphocyte Apoptosis

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THE INDIRECT EFFECT OF BVDV ON IMMUNE SUPPRESSION: THE ROLE OF INFECTED MACROPHAGES IN LYMPHOCYTE APOPTOSIS

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This thesis is approved as creditable and independent investigation by a candidate for the Master of Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that conclusions reached by the candidate are necessarily the conclusions of the major department.

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LIST OF ABBREVIATIONS

%: Percentage

°C: Degree centigrade

µg: Microgram

µl: Microliter

APC: Antigen presenting cells

B-cell: B lymphocyte

BD: Becton, Dickinson

BM: Bone Marrow

Bt cell: Bovine turbinate cells

BVD: Bovine viral diarrhea

BVDV: Bovine Viral Diarrhea Virus

C: nucleocapsid protein

CD14: Cluster of differentiation 14

CD11b: Cluster of differentiation 11b

CO2: Carbon dioxide

CP: Cytopathic

CPE: Cytopathogenic effect

ELISA: Enzyme-linked immunosorbent assay

FBS: Fetal Bovine Serum

FITC: Fluorescein isothiocyanate

Gp: Glycoprotein

h: Hour

IACUC: Institutional Animal Care and Use Committees

IFA: Indirect fluorescence assay

IFN: Interferon

IgG: Immunoglobulin G

IgM: Immunoglobulin M

IHC: Immunohistochemistry

IL: Interleukine

kD: Kilodalton

Log: Logrithum

LPS: Lipopolysaccharide

MDBK: Madin Darby bovine kidney cells

MDDC: Monocyte-derived Dendretic cells

MDM: Monocyte-derived macrophages

MEM: Minimal essential medium

mg: Milligram

MHC I: Major histocompitability class I

MHC II: Major histocompitability class II

min: Minute

ml: Milliliter

MLV: Modified live vaccine

MOI: Multiplicity of infection

NCP: Non-cytopathic

Ng: Nano gram

NS: Non structural protein

PI: Persistent infection

PBMC: Peripheral mononuclear cells

PBS: Phosphate buffered saline

p.i: Post infection

PI: Persistent infection

Q-PCR: Quantitative polymerase chain reaction

RNA: Ribonucleic acid

RPMI: Roswell Park Memorial Institute (Medium)

RT-PCR: Reverse transcriptase polymerase chain reaction

Sup: Supernatant

T cell: T lymphocyte

TNF: Tumor necrosis factor

U: UNIT

UTR: Untranslated region

UV: Ultraviolet

VMRD: Veterinary Medical Research & Development

µl: Microliter

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ABSTRACT

THE INDIRECT EFFECT OF BVDV ON IMMUNE SUPPRESSION: THE ROLE OF INFECTED MACROPHAGES IN LYMPHOCYTE APOPTOSIS

KARIM ABDELSALAM

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BVDV is an important pathogen of cattle that affects both the dairy and beef industry causing severe economic losses. The main problem of BVDV is the immune suppression that is associated with lymphoid depletion shortly post infection. The mechanism of this immune suppression is not well understood. In this *in vitro* study, we sought to determine the indirect effect of BVDV infection in macrophages as a key player in the pathogenesis of BVD especially, the apoptotic effect induced on lymphocytes. We infected bovine monocyte-derived macrophages (MDM) with either high or low virulent strains of cytopathogenic or non-cytopathogenic strains of BVDV and collected supernatants that were used for treatment of the fresh peripheral blood lymphocytes or BL-3 B-cell line. We found that only supernatant from the high virulent BVDV strain induced lymphocyte apoptosis. Furthermore, direct infection of lymphocytes with the same virulent strain did not induce significant apoptosis. We investigated the apoptosis-related cytokine profile of the infected macrophages by qRT-PCR with different BVDV strains. There was no significant difference in the transcriptional profiles between strains so cytokine induction does not seem to be a mechanism of induced apoptosis. We then investigated the role of possible secreted viral proteins as the mechanism of indirect apoptosis of lymphocytes by treating the supernatants with specific antibodies against BVDV and its proteins. These treated supernatants still had lymphocyte apoptosis

activity, ruling out their responsibility as well. Our data suggest an important role of macrophages in the mechanism of lymphocyte depletion by high virulent strains of BVDV, however further studies are required to determine the identity and mechanism of action of these apoptotic factors present in the supernatant of the infected macrophages.

CHAPTER 1

RESEARCH OBJECTIVES AND LITERATURE REVIEW

INTRODUCTION

BVDV is a viral pathogen of cattle and other ruminants that threatens both the dairy and beef industry worldwide causing severe economic losses. Immunosuppression attributed to lymphoid depletion is a major problem associated with BVDV. BVDV associated lymphoid depletion following infection with highly virulent strain of BVDV is often irreversible and leads to high mortality. The immunosuppression associated with BVDV also interferes with the responses to vaccination. This BVDV associated immunosuppression is not well-studied and further investigation still required.

RESEARCH GOAL AND OBJECTIVES

- The long-term goal of this research is to provide new insight about viral pathogenesis and viral-host relationship for better understanding of the immune suppression associated with BVDV. This was achieved by the following objectives:
    1. Investigate the direct and indirect effect of a highly virulent NCP BVDV strain on total lymphocyte populations.
    2. Investigate the apoptosis-induced-secretory-properties in the supernatant of the macrophage infected with highly virulent NCP BVDV.
    3. Perform cytokine transcriptional profiling of macrophages infected with highly virulent BVDV strains.

LITERATURE REVIEW

1. Biology of Bovine Viral Diarrhea Virus (BVDV)

1.1. BVDV Definition and Economic Importance

Bovine viral diarrhea virus (BVDV) is a pathogen that predominately affects cattle throughout the world (Meyling et al., 1990). The emergence of more virulent forms of the virus in the past few decades have made this a pathogen of increasing economic importance (Goens 2002).

The overall effects of BVDV infection are difficult to examine, due to the breadth of the disease. Research and data highlighting the effects of the disease is very limited, simply due to the fact that too many variables are present for accurate measurement in addition to lack of knowledge about its pathogenesis. Furthermore, with the worldwide prevalence of BVDV, it is easily understood why BVDV infections have a huge economic impact (Larson et al., 2004).

BVDV is a pantropic viral infection that affects reproduction and production in addition to immunosuppression. Reproductive disorders caused include abortion, early embryonic deaths, and fetal defects. Production effects are associated with decline in feed-to-gain efficiency, lower carcass quality, and decreased milk production (Hafez, 1975). More importantly is the immune dysfunction caused by BVDV and consequently, secondary bacterial infection and vaccination failure that will lead to continual spread of the virus. The virus' high prevalence rate, resultant abortions, persistent infections and manifestations (i.e. mucosal disease) make prevention and active management of BVDV essential for cattle producers (Ridpath, 2013).

Despite the current vaccine control strategy that has been used for over 60 years, significant economic losses from BVDV are still reported in the United States (Ridpath, 2012) and worldwide (Stahl and Alenius, 2012). BVDV infection of circulating white blood cells may change their ability to stimulate adaptive immune responses and facilitate chronic infection of these cells, thus contributing to the failure of the control strategy (Givens and Marley, 2013). Previous studies on the economic impact of this virus on cattle industry indicate that the estimated economic losses range up to $80 per head (Table 1).

1.2. BVDV structure

BVDV is a small, enveloped, single-stranded, positive sense RNA virus of about 45-50 nm in diameter. The morphology of BVDV is pleomorphic but mainly spherical (Coria et al., 1983; Collett, 1992). BVDV has an icosahedral capsid that is surrounded by tightly adherent lipid bilayer envelope (5-7nm thickness) carrying on its surface glycoprotein spikes (Figure 1.1.) (Thiel et al., 1996).

BVDV genome is a single positive sense infectious RNA with a single open reading frame (ORF) bounded by 5' and 3' un-translated region (UTR). The actual length of the virus genome is variable due to changes in the ORF region. The NCP SD-1 strain is 12.3 kb in length (Collett, 1992) while the length of CP NADL and Osloss strains is 12.5 kb. These changes in the size of ORF have different forms including insertion, deletion, rearrangement and duplication (Purchio et al., 1984).

The ORF encodes a single polyprotein of 3888 and 3998 amino acids for NCP and CP biotypes respectively. This large polypeptide is proteolyticly processed during virus replication to produce a variety of viral structural and nonstructural proteins

(Murphy et al., 1999). These proteins beginning on the 5' third of the virus include one nonstructural protein; P20/N^{PRO} followed by four structural proteins; P14/C, gp48/E0, gp25/E1, gp53/E2, and the last three structural proteins forms envelope glycoprotein spikes. The remaining of the ORF is translated to give other nonstructural proteins (Figure 1.2.) (Deng and Brock, 1992).

1.2.1. The proteins encoded by BVDV genome:

The viral encoded proteins depend on the biotype of the virus: CP BVDV isolates possess both the P80 and P125 while NCP display only P125 (Akkina, 1982). The genome of the BVDV has a large ORF with a capacity to encode structural and nonstructural polyprotein of 3898 amino acids and of a 438 kD protein (Deng and Brock, 1992). A total of 10-13 virus-induced polypeptides have been identified in infected cells and CP BVDV express one or more additional polypeptides (Collett et al., 1989)

Npro/P20 Npro is the first translation product of the ORF. This protein consists of 168 amino acids, with an observed mass of 20 kD in SDS-PAGE. It is a cis-acting papain-like protease that cleaves inter-molecularly at its C-terminus, releasing itself from the nascent poly-protein. Npro then cleaves the large poly-protein into many structural and nonstructural viral proteins. The functions of this papain-like protease in virus replication include generation of the terminus of C (Rumenepf et al., 1998), as well as specific blocking of the activity of interferon regulatory factor 3 (IRF-3), a transcription factor essential for interferon promoter activation and so inhibition of IFN antagonistic action on virus replication (Hilton et al., 2006)

C/P14 It is the second protein encoded and the first structural protein that comprise the capsid protein of 102 amino acids weighting 14 kD. It is a well-conserved

protein in most pestiviruses. The function of this protein is to package the genomic RNA and to provide necessary interaction for formation of the enveloped virion. This protein is weakly antigenic in cattle, as sera from convalescent cattle doesn't contain antibodies to C (Donis, 1995). However, when a recombinant adenovirus containing C was injected in mice, high levels of humoral and cellular immune responses against C protein were produced, indicating highly immunogenic in the mouse (Elahi et al., 1999).

E^{rns}/E0/gp48 It is a glycoprotein of 227 amino acids with a MW of 44-48 kD for the intracellular anchor protein, however another secreted form of E^{rns} glycoprotein was detected while migration on SDS PAGE with a molecular weight of 46-69 kD due to glycosylation . E^{rns} represents an unusual type of membrane anchor. Analyses with different extraction procedures showed that E^{rns} is neither easily stripped from the membrane, like a peripheral membrane protein, nor as tightly membrane bound as a transmembrane protein (Fetzer et al., 2005). The function of E^{rns} is unclear but it appears to have RNase activity and is thought to cleave RNA inside the cell. The entire E^{rns} protein is able to translocate into the cell. Furthermore, other proteins and even active enzymes could be transported inside the cell when they were attached to the C-terminal E^{rns} peptide (Langedijk, 2002). This protein is highly conserved among different pestiviruses. Although this structural protein is highly immunogenic, it produces limited virus neutralizing antibody activity (Donis, 1995). On the other hand, E^{rns} glycoprotein of CSFV can be secreted and induce apoptosis in lymphocyte populations (Bruschke et al., 1997). The variation in E^{rns} glycoprotein will lead to diagnostic failure of BVDV by different diagnostic techniques as indirect fluorescence assay (IFA), and enzymelinked immunosorbent assay (ELISA) (Gripshover et al., 2007).

E1/gp25 This glycoprotein has a MW of 21–25 kD and is ~195 amino acids in length. This protein has 2 hydrophobic transmembrane portions that initiate translocation of the adjacent polypeptide, E2. E1 is covalently linked with E2 by disulfide bonds (Donis, 1995). The antibodies produced against this glycoprotein are not protective as the level of antibodies against this protein in convalescent cattle were not significant. However, this protein elicited monoclonal antibodies in mice (Kreutz et al., 2000).

E2/ gp 53 This glycoprotein is ~375-400 amino acids long and has a MW of 41-45 kD. It contains three to four consensus sites for glycosylation and it is anchored in the envelope as homo and heterodimers with E1. E2 is highly antigenic as it provokes the production of neutralizing antibodies in the host after infection or vaccination (Donis, 1995).

NS2-3/P125 It is the biggest nonstructural protein and is a serine protease. It has a MW of 125 kD and is composed of ~1300 amino acids. This protein is 2 polypeptides: NS2 and NS3 (Donis, 1995). This protein plays an important role in virus replication as well as induces a strong humoral immune response against it. The humoral immune response induces a response to live attenuated vaccines but not against killed vaccines, which is unable to replicate, suggesting its important role in replication. This complex protein aided in the inhibition of IFN-beta production inside the cell. This inhibition of the major anti-viral mechanism allows the rapid spread of the NCP strain and certainly is one of the reasons that NCP BVDV is the major biotype in nature (Paton et al., 1991).

NS2/P54 This polypeptide is a product of the cleavage of NS23 and composed of 400 amino acids with a MW of 54 kD. This polypeptide doesn’t exist in all cytopathic

isolates of BVDV (Donis, 1995). It is poorly immunogenic and doesn't induce humoral immunity in infected cattle (Bolin and Ridpath, 1989).

NS3/P80 It is a polypeptide of 80 kD found only in cytopathic strains, and is considered a marker for cytopathogenicity. It is the most conserved protein in the genus pestiviruses, it is very stable in infected cells and of high antigenicity (Donis, 1995). This polypeptide is a multi-functional protein that helps in RNA replication and energy production (Gu et al., 2000). Expression of NS3/4A resulted in increased activity of caspase-9 and caspase-3, indicating induction of the intrinsic apoptosis pathway. A mutation in NS3/4A without protease activity was unable to induce apoptosis, suggesting that NS3 protease activity is required for initiation of apoptosis during CP BVDV infection. This polypeptide is unable to inhibit TLR3- and RIG-I-dependent activation of the IFN-beta promoter (Gamlen et al., 2010).

NS4A/P10, This polypeptide is composed of 55 amino acids with a MW of 10 kD. It is not immunogenic but seems to be well conserved among different pestiviruses suggesting important role in replication (Donis, 1995). NS4A act as co-factor for proteinase enzymatic activities of NS3 (Gamlen et al., 2010)

NS4B/P32, It is a polypeptide of 32 kD with a regulatory role in the protease activity of NS23 in the generation of NS5B. It is not immunogenic, which is confirmed by failure of infected cattle to produce NS5B antibodies (Donis, 1995). Induction of a mutation in NS4B can attenuate BVDV cytopathogenicity despite NS3 production, suggesting an important role in the replication of CP strains (Qu et al., 2001).

NS5A/P58, This polypeptide contains ~450 amino acids and has a MW of 58 kD. NS5A is a phosphoprotein which has several roles in the Flaviviridae replication and

cytopathogenicity (Majumder et al., 2001). Pestiviruses have split their RNA polymerase activity into two polypeptides; NS5A and NS5B. No humoral immune response is evoked against this protein in either infected or vaccinated animals (Donis, 1995).

NS5B/P75, This polypeptide has a molecular weight of 75 kD. It is the last encoded nonstructural protein and has a putative viral RNA-dependent RNA polymerase. It has the shortest half-life of all BVDV polypeptides. No serum antibodies can be detected against this polypeptide (Zhong et al., 1998)

1.3. BVDV Taxonomy and Classification

BVDV has been recently classified in unassigned order, Family: *Flaviviridae*, which contain 4 genera; *Flavivirus, Hepacivirus, Pegivirus and Pestivirus*, the latter genus includes BVDV (ICTV 2015).

Genetic sequencing and analysis of 5' UTR of BVDV viral genome has determined that there are two genotypes: type I and type II. BVDV type II lack an internal Pstl restriction site that is present in 5' UTR of all BVDV type I isolates (Elahi et al., 1999). Also, BVDV type I shows greater intragenotypic variability than BVDV type II (Kwang et al., 1991; Lewis et al., 1991) (Figure 1.3.) and can be subdivided into many subtypes BVDV (1a - 1u), while BVDV type II can be subdivided into only three subtypes (2a - 2c) based on the difference in N^{pro} and E2 sequences (Strong et al., 2012;Liu et al., 2009; Mao et al., 2016). An unclassified atypical pestiviruses referred to as Hobi-like BVDV (BVDV type III) has been identified that is phylogenetically distant from BVDV-1 and BVDV-2. This virus is also antigenically distinct so BVDV-1 & -2 diagnostics and control strategies will be ineffective (Figure 1.3.) (Bauermann et al., 2013).

Each genotype can be further subdivided into 2 biotypes: cytopathic and non-cytopathic strains according to its pattern of growth in cell culture. Most of the BVDV isolates obtained from field cases are NCP in cell culture (Brock, 1995). On the other hand, CP virus is associated with mucosal disease in PI animals; this will occur after the superinfection with CP strain of PI animals, leading to fatal mucosal disease (Werdin et al., 1989; Greiser-Wilkie et al., 1993). Recently another biotype of BVDV, lymphocytopathogenic has been identified. These are NCP strains that induce apoptosis in lymphocyte populations (Ridpath et al., 2006).

2. BVDV replication

2.1. Overview of virus replication

BVDV replicates efficiently in cells derived from order *Artyodactyla*, reflecting the *in vivo* host range of the virus. Optimal replication takes place in bovine, ovine or caprine cells, whereas replication is less efficient in swine cells (Bolin et al., 1994). The replication of NCP-BVDV in cell culture of bovine origin causes no changes in these cells (Moennig and Plagemann, 1992). Rabbits are susceptible to infection with rabbit-adapted BVDV (Fernelius and Lambert, 1969). Mice also can be infected with BVDV by intra-nasal and intra-peritoneal routes and the virus can be detected in spleen, lymphoid tissue, the lungs and the stomach 7 days post infection but not in the liver and kidney (Seong et al., 2015).

BVDV is one of the RNA viruses that is characterized by intracytoplasmic replication with production of new progeny within 12 to 14 hours from the start of infection (Moennig, 1990). Each infected cell produces 100 to 1000 complete infectious

virions (Donis, 1995). Not all susceptible cells will release new progeny. Bovine macrophages are a good example; these cells are infected and the virus replicates with new viral protein formation, but they fail to produce new progeny. The block in production may be associated with the assembly or the release of the virus (Chase et al., 2015)

2.2. Attachment and Penetration:

The initial trigger of endocytosis is the E2 spike interacting with a 50 kD cellular receptor present on healthy susceptible cells (Xue and Minocha, 1993). It is important for the susceptible cell not to be treated with any materials such as proteases as the latter make the cells resistant for infection. The process of endocytosis is mediated by low density lipoprotein (LDL) receptors on permissive cells as complete inhibition of this kind of receptors will consequently the process of penetration. Further evidence is provided by lack of LDL receptors on BVDV resistant cells (Agnello et al., 1999). The E^{rns}, E1, and E2 have important roles in virus binding and cell entry as well as for immunologic recognition by the host. The E2 protein contains the major recognition sites for BVDV neutralizing antibodies against BVDV. The neutralizing epitopes of E2 are important targets for BVDV vaccine efficacy (Chimeno Zoth et al., 2007). Unlike E1 and E2, E^{rns} is dispensable for cellular entry (Ronecker et al., 2008). The E1 protein is predicted to have various functions such as a membrane anchor for E2. The E1 and E2 form E1-E2 heterodimers. The E1-E2 heterodimers appears to be essential for cell entry of BVDV (Rumenapf et al., 1993; Ronecker et al., 2008). After the process of entry, uncoating of viral nucleic acid is achieved by the aid of acidic pH of cellular endosome (Boulanger et al., 1992).

2.3. Gene translation:

Translation of the viral genome requires partial stripping of the capsid protein C from the RNA to start translation of the virus-encoded genes in the infected cells. The process of translation begins when 5' UTR, which act as an internal ribosomal entry site (IRES), that binds independently to ribosomal 40s subunit and eukaryotic initiation factor (eIF) 3. Another important thing is that the 5' UTR contain determinants that mediate direct attachment of 43S ribosomal complexes to the initiation codon (Pestova and Hellen, 1999). The translation process yields a single polyprotein that can be detected in foetal bovine testicles cells as early as 3 hours after infection, reaching a peak at 12 to 14 hours post infection (Donis and Dubovi, 1987). The 5' UTR encodes a bi-functional secondary structure motif that enables the viral RNA to switch from the translation to the replication cycle and vice versa (Yu et al., 2000 and Li and McNally, 2001).

2.4. Processing of BVDV polyprotein:

The polyprotein is subjected to 9 or 10 cleavage events that yield 4 structural and 8 nonstructural proteins (Wiskerchen and Collett, 1991; Akkina, 1991). Several regulatory and catalytic intermediates are produced during polypeptide processing (Chambers et al., 1990). The processing of the polyprotein starts with cleavage of nucleocapsid protein (C) with Npro and E^{rns}. Later, the amino-terminal end of C protein is cleaved by a co-translational autoproteolytic event by Npro (Stark et al., 1993). This is followed by cleavage of the C protein from E^{rns} in the host cell's endoplasmic reticulum. The other structural proteins are cleaved by cellular proteases (Ronecker et al., 2008).

2.5. BVDV genome replication:

Once the viral enzymes necessary for RNA replication are produced by translation of BVDV genomic RNA and polyprotein processing, the viral life cycle enters its RNA genome replication phase. Two well-defined processes are required to produce identical copies of the incoming plus sense genomic RNA. First, a negative strand copy is made to serve as template. Second, progeny viral RNA is synthesized using the template from the previous step. Additional polypeptides are likely to participate in RNA replication (Donis, 1995).

Experimental data demonstrate that the enzymatic activities located in C-terminal portion of the NS3 protein, particularly the ATPase/RNA helicase, play a pivotal role during early steps of the viral replication pathway (Gu et al., 2000). The prototype replicon, BVDV D19c, containing the genomic 5' and 3' UTR and truncated ORF encoding mainly the nonstructural proteins NS3, NS4A, NS4B, NS5A and NS5B was used to study which proteins were essential for viral replication and whether they acted in cis or in trans. Introduction of a large spectrum of in-frame mutations into the D19c ORF was done. This suggested that the region from NS3 to NS5B played a pivotal role even during the early steps of the viral replication pathway. Replication of functional and nonfunctional NS5A mutants could be clearly enhanced and restored respectively (Grassmann et al., 2001).

2.6. Assembly and release:

With the packaging of progeny RNA and assembly of virions, the replication cycle enters its final stages. Signals for RNA encapsidation by C protein haven’t been defined, however RNA binding sites on the C protein with specific structures on the viral RNA

was considered necessary. Evidence from electron microscopic studies suggests that BVDV assembly takes place in the ER or the Golgi. During this process, that likely involves contact between C and the tail of E2, virions acquire a lipid envelope and accumulate transiently in the lumen of vesicular transport (Donis, 1995). The BVDV envelope glycoproteins, E^{rns} and E2, as well as the nonstructural proteins, NS2-3 and NS3, are highly associated with intracellular membranes. These finding indicate that BVDV is released by budding into the cisternae of the endoplasmic reticulum (Grummer et al., 2001).

3. Bovine Viral Diarrhea Virus Disease

3.1. BVDV infection strategies

BVDV is considered a successful virus from the epidemiological point of view because of its ability to persist. This success leads to its worldwide distribution and high rate of infection. The secret behind this success is a combination of two well-known strategies, the “hit-and-run” strategy and the “infect-and persist” strategy. BVDV has the ability to use a combination of both strategies in nature.

3.1.1. Hit and Run

The hit and run strategy is the most common strategy used by the virus. This strategy involves a short period of susceptibility to infection in their host so that it can multiply and survive, leading to a transient infection. Following the multiplying of the virus the hosts develop antibodies to protect them from a repeat BVDV infection for the rest of their relatively short life. By this strategy the virus relies on continuously finding new susceptible hosts (Figure 1.4.) (Schweizer et al., 2006).

3.1.2. Infect and Persist

The hit & run strategy is considered insufficient for the survival of BVDV, in other words, without PI animals, the virus will disappear. BVDV has the ability to persist in its host. The persistent infection caused by BVDV results when the non-cytopathogenic virus evades the immune system of the host by infecting the pregnant animal and then the fetus at the first trimester of pregnancy specifically from 30-110 days of gestation prior to attainment of immune-competence. The fetus develops an immunotolerance towards the virus (Figure 1.4.) (Ridpath, 2012).

3.2. BVDV Pathogenesis and Disease outcomes

Viral pathogenesis is the sum of its effects on host cellular protein function, gene expression and the capacity of the host cell to maintain its integrity (Lyles, 2000). BVDV is characterized by complex pathogenesis that is reflected in the wide variety of clinical signs (Brownlie et al., 1984). Titration of different strains of BVDV on bovine, ovine and porcine cells showed that pestiviruses infected bovine, ovine and porcine in a like manner (Meyers et al., 1992).

During the acute infection, the initial infection takes place in the oronasal mucosa and the virus spreads from this site systemically. BVDV invades the lymphoid tissue, and disables the recognition of effector functions of immunity with subsequent capability of persistence (Brownlie, 1990). Field strains capable of rapid growth in the oronasal mucosa have been involved in the acute disease. Systemic spread of infection may occur as free virus in serum or as virus associated with the cells in the blood; the lymphocytes and monocytes are generally regarded as being particularly sensitive to BVDV infections (Bruschke et al., 1998).

After infection, BVDV antigen can be recovered from nearly all tissues and different biotypes can be isolated from different sites, whether it is mucosal disease, acute infection or persistent infection. However, in the case of mucosal disease, large amounts of BVDV antigen were detected mainly in tissue samples from lymphoid and GI tissue, with only small amounts in the respiratory tract. Generally, abundant NCP BVDV antigen was detected in the pituitary gland, and in pancreatic Langerhans islets, whereas cytopathic BVDV was isolated mainly from mesenteric lymph nodes and Peyer's patches (Spagnuolo et al., 1997).

There is a difference in the virulence and pathogenesis of the BVDV that appears to be strain and biotype dependent (Bolin and Ridpath, 1992). Both CP and NCP BVDV biotypes were isolated from herds experience acute and chronic BVDV.

The two biotypes of BVDV also play an important role in creating MD (Brownlie, 1991). When the PI animal, initially infected with NCP biotype at early gestation, becomes superinfected with CP strain leading to initiation of mucosal disease. This can be ther result of spontaneous mutation from NCP to CP biotypes leading to development of MD (Baker, 1995; Darweesh et al., 2015)

The degree of antigenic homology between the CP and NCP strains may be an important factor in determining the pathogenesis of MD (Meyers et al., 1989, and Shimizu et al., 1990). Acute fatal MD occurs when PI cattle are exposed to CP biotype of the same genotype, that is to say homologous biotypes, however if the PI cattle are exposed to a heterologous CP strain of different genotype, chronic MD may develop instead (Baker, 1995). The level of circulating gamma-delta T cells might also determine whether or not PI animals develop MD (Bruschke et al., 1998).

The type of clinical response to infection with BVDV is variable and multifactorial. It depends on several factors such as, the immune status, pregnancy status and the age of the fetus as well as concurrent level of environmental stress (Bolin and Ridpath, 1992).

3.3. BVDV-induced Immunosuppression:

The virus appears to be able to replicate in all of the major lymphocyte subpopulations as well as in accessory cells. This may result in the leukopenia that is often a sequel of infection and affects B-cells as well as the T-cell sub-populations expressing either BoCD4 or BoCD8 antigens. B and T-cell responses are affected as a consequence of exposure to BVDV and there is a reduced ability to control other infections. Although the fetus can mount an immune response in the latter part of gestation, during the first trimester it does not. A specific state of tolerance is induced and this is associated with change in the proportion of certain lymphocyte subpopulations and ability to respond to immune stimulation (Howard, 1990).

Non-cytopathic BVDV fails to induce interferon type I in cultured bovine macrophages whereas cytopathic biotypes readily trigger this response. Cells infected with non-cytopathic BVDV are also resistant to induction of interferon by double stranded RNA, a potent interferon inducer signaling the presence of viral replication in the cell (Schweizer and Peterhans, 2001). Thus, non-cytopathic BVDV may dispose of a mechanism suppressing a key element of the antiviral defense of the innate immune system. Since interferon is also important in the activation of the adaptive immune response, suppression of this signal may be essential for the establishment of persistent infection and immunotolerance (Peterhans et al., 2003).

The level of suppression of the adaptive immune response is strain-dependent. Non-cytopathic (NCP) BVDV infection stimulates cytokines from macrophages *in vitro* but the effect of BVDV infection *in vivo* on macrophages or *in vitro* with monocytes is not clear. Antigen presentation is decreased and co-stimulatory molecules are down regulated. T-lymphocytes numbers are reduced following BVDV infection in a strain-dependent manner. There is recruitment of lymphocytes to the bronchial alveolar space following cytopathic (CP) BVDV infection. Depletion of T-lymphocytes occurs in the lymphoid tissue and is strain dependent. BVDV CP T-lymphocyte responses appear to be primarily a T helper 1 response while the response following NCP BVDV induces a T helper 2 response. (Chase, 2013).

Many immune cells are susceptible to BVDV infection including monocyte and granulocytes. This infection is associated with reduction of their functions. BVDV infection leads to severe reduction in lymphocyte population as it affects both thymic and follicular T-lymphocytes (Brewoo et al., 2007).

Acute BVDV infection or vaccination with modified live BVDV vaccine results in decreased delayed type hypersensitivity T cell response to *Mycobacterium avium and Mycobacterium paratuberculosis* purified protein derivative (Thoen and Waite, 1990). Infection of calves with non-cytopathic viral diarrhea virus (BVDV) resulted in the temporary suppression of lymphocyte proliferation and interferon-gamma production leading to vaccination failure against other infectious agents (Charleston et al., 2001). MLV BVDV vaccination resulted in the inhibition of antibody-dependent cellular cytotoxicity by neutrophil (Roth and Kaeberle, 1983). The BVDV-infected monocytes

reduced leukotriene B4, an important mediator for the production of some pro-inflammatory cytokines as IL-1, IL-2 and IFN-alpha (Atluru et al., 1992).

Unlike NCP strains, CP BVDV up-regulate both IFN type I and IL-12 within 1 h post infection of monocyte. Both IFN-γ and IL-12 are secreted by NK cells and T cells. The IL-12 acts as a growth factor for activated NK cells and T cells and enhances the cytotoxic activity of NK cells and favors cytotoxic T lymphocyte generation (Abbas et al., 1996; O'Garra, 1998; Trinchieri, 1995; Kalinski et al., 1999).

4. BVDV Immune Response

4.1. Immunology to natural infection:

Infection of normal calves with bovine virus diarrhea virus (BVDV) is a transient self-limiting infection that can result in a period of immunosuppression (Howard, 1990). Both humoral and cellular immune responses following natural infection are protective. The majority of the B cell response (as measured by serum antibodies) is directed against the viral proteins E2 and NS2/3, with minor responses against the E^{rns} and E1 proteins. The major neutralizing epitopes are conformational and are located within the N-terminal half of the E2 protein (Ridpath, 2013).

E2 induces neutralizing antibody in experimental and natural hosts. Moreover, neutralizing antibodie raised against E2 are able to protect susceptible hosts from infection with BVDV (Ferrer et al., 2007; Rosas et al., 2007; Thomas et al., 2009). The induced antibodies against BVDV result in a 4-fold rise 2-4 weeks post infection. The response is detected at a peak 8 to 10 weeks up to 1 year after infection then the antibody levels decrease very slowly (Howard, 1990). The heterogeneity that exists among circulating BVDV strains prevents protective cross immunity (Ridpath, 2013).

4.2. BVDV maternal immunity:

Calves receive maternal antibodies via colostrum uptake. In the first weeks of life, these antibodies prevent a BVDV infection. Protection lasts approximately 6 months, and even as long as 9 months (Munoz et al., 2002). Passive maternal antibodies protect against nasopharyngeal shedding of the virus, and reduce leukopenia in challenge inoculated calves (Howard et al., 1989). The mean half-life of maternal antibodies in non-vaccinated calves to BVDV1a, BVDV1b, BVDV2 was 23.1, 22.8, 22.9 days respectively. The mean half-life of viral antibodies was greater for vaccinates than for nonvaccinates for BVDV. The calculated mean time to seronegative status for nonvaccinates based on titers at day 0 was: BVDV1a, 192.2 d; BVDV1b, 179.1 d; BVDV2, 157.8 days (Fulton et al., 2004).

Interference by maternal antibodies prevents the development of an antibody response following vaccination with most of killed and attenuated BVDV vaccine with exception to adjuvant based killed vaccines. However, T cell mediated immune response to BVDV may be generated in the absence of a detectable serum neutralizing antibody response. Vaccinating young calves against BVDV while maternal antibody is present may generate BVDV specific memory T; CD4+, CD8+, and gamma delta T cell responses as well as B cells. Seronegative calves with memory T and B cells specific for BVDV may be immune to challenge with virulent BVDV (Endsley et al., 2003; Zimmerman et al., 2006; Platt et al., 2009).

4.3. Immunology to vaccinated animals:

Both modified live and killed vaccines are efficacious under controlled conditions. Both humoral and cellular immune responses are protective. Following natural infection or vaccination with a modified live vaccine, the majority of the B cell response, as measured by serum antibodies, is directed against the viral proteins E2 and NS2/3, with minor responses against the E^{rns} and E1 proteins. Thus, E2 glycoprotein constitutes an excellent candidate for the development of an experimental subunit vaccine. Vaccination with killed vaccines results in serum antibodies directed mainly at the E2 protein. Vaccination, while not 100% effective in every individual animal, is effective at the herd level (Ridpath, 2013).

4.4. Immunology of PI animal:

PI animals develop neither neutralizing nor non-neutralizing antibodies against BVDV, due to specific B and T lymphocytes immunotolerance (Larsson and Fossum, 1992). Sometimes, PI animals develop low titers of antibody that continue to decrease with time. The presence or absence of neutralizing antibody in PI animals probably based on the stage of pregnancy and the involvement of maternal previously present maternal antibodies (Kane et al., 2015). However, it is considered as immunocompetent against other viruses (Houe and Heron, 1993). Ingestion of colostrum to PI animal seems not to be helpful any more rather it interferes with the initial isolation of the virus. Passively derived BVDV antibodies decrease more rapidly in PI calves than in immunocompetent. It was shown that this immunotolerance is highly specific: immunotolerant animals, upon infection with a sufficiently different type of BVD/MD virus, develop an immune response against that virus without, however, being protected against their “own” virus,

i.e. the immune system is capable of differentiating between sufficiently distinct types of virus (Chase et al., 2004). BVDV uses the macrophage/dendritic cell as a 'lymphocyte hitman' that will help with lymphocyte apoptosis (Chase et al., 2015).

4.5. BVDV associated lymphoid apoptosis

BVDV is associated with immune suppression due to lymphoid depletion that can be mild to severe, with variable levels of recovery depending on the virulence of the BVDV strains (Ammari et al., 2012; Falkenberg et al., 2014; Liebler-Tenorio et al., 2003). Interestingly, this lymphoid depletion was associated with increased number of macrophages 3 days post infection in lymphoid tissues. The presence of this significant number of macrophages in the lymphoid tissues suggests an indirect effect of this cell on the lymphoid depletion caused by BVDV (Pedrera et al., 2009).

Previously, the same scenario has been proposed for the pathogenesis of classical swine fever virus (CSFV), another member of the pestivirus group in the Flaviviridae. CSFV can induce lymphoid apoptosis directly and indirectly via macrophages. With CSFV, TNF-a production has induced apoptosis (Choi et al., 2004). The E^{rns} protein of CSF is responsible for lymphocytic apoptosis (Bruschke et al., 1997). Recently, the involvement of apoptosis-induced cytokines, TNF-a and IL-1a, have been ruled out from BVDV pathogenesis (Pedrera et al., 2009). Furthermore, the role of macrophages could be only restricted to phagocytosis of apoptotic cell debris (Raya et al., 2012). Other groups have suggested that most of the cells undergoing apoptosis after CP BVDV infection are monocytes. Also CP BVDV can induce apoptosis in purified CD4 T lymphocyte, which was enhanced by the presence of monocyte in a mixed population

unlike CD8 T cells that undergo apoptosis only in a mixed population with monocyte (Lambot et al., 1998).

Generally, there are 2 main pathways for induction of apoptosis: intrinsic and extrinsic pathway, The apoptosis induced by cytopathic BVDV is associated with the intrinsic but not the extrinsic pathway, especially due to the lack of caspase-8 expressio,n which is the main mediator for extrinsic pathway whereas caspase-9, which is important mediator of intrinsic pathway, was strongly expressed (Hilbe et al., 2013). Besides the two biotypes of BVDV, CP and NCP, a third biotype has been proposed, which is lympho-cytopathogenic and is associated with highly virulent strains. This third biotype is includes highly virulent NCP biotypes that don't induce CPE on cultured susceptible cells but induce apoptosis in cultured lymphoid cells (Ridpath et al., 2006). There are many differences present between the 2 biotypes of BVDV that may affect the mechanisms of viral pathogenesis. There are around 26 genes differentially expressed between CP and NCP BVDV in the infected cell (Werling et al., 2005). Cytopathogenic BVDV exert its cytopathic effect on the susceptible cells via apoptosis, moreover, it has been proposed that this apoptosis is associated with the direct effect of the virus on the infected cell rather the interaction between infected and normal cell. Moreover, only CP but not NCP strains can induce direct apoptosis to PBMCs (Zhang et al., 1996)(Lambot et al., 1998).

Previous literature has proposed many hypotheses for the apoptotic ability of cytopathogenic BVDV. Some researchers suggested the involvement of NS3 as one of the main difference between CP and NCP BVDV. This is along with higher levels of RNA accumulation in CP BVDV-infected cells than NCP BVDV-infected cells (Vassilev

and Donis, 2000). The correlation between apoptosis and cytopathogenic effect of the virus was confirmed later by other group that discussed the role of apoptosis inhibitors in delaying the cytopathogenic effect of BVDV (Grummer et al., 2002).

Unlike CP BVDV, the NCP BVDV inhibits both IFN I synthesis and RNA accumulation and the subsequent induced apoptosis in the infected cell. It has been thought that this inhibition is mediated through the specific interfering with the intracellular action of accumulated ds RNA but not with its uptake into the cells (Schweizer and Peterhans, 2001).

Interestingly, the infection of monocyte/macrophage with CP BVDV leads to up-regulation of crucial chemokines of CCL and CXCL families that are important in induction of proper immune response against the infection. In contrast. NCP BVDV down-regulated such important chemokines (Burr et al., 2012). Proper activation of PBMCs in response to BVDV infection can protect them against apoptosis via TRAF2 dependent CD 28-4-1BB signaling (Song et al., 2016).

4.6. Immune cells associated with this BVDV study

This study was concerned with determining the direct and/or the indirect effect of BVDV on lymphocyte populations. To study the indirect effect, the monocyte-derived macrophages (MDM) were infected first with different strains of BVDV and the irradiated infected supernatant were incubated with lymphocyte populations that were collected from the blood or obtained from bovine lymphosarcoma cell line (BL-3).

4.6.1. Bovine monocyte derived macrophages (MDM)

Bovine macrophages are an important antigen presenting cell that is responsible for uptake of infectious agents and processing the antigen for the effector immune cells to

exert its specific immune mechanism to clear the infection. Macrophages originate from hematopoietic stem cells and myeloid progenitor cells present in the bone marrow. Monocytes, the progenitor for macrophages develop from myloid stem cells in the bone marrow. The monocytes traffic from into the tissues to differnetiate into professional macrophages (Chase et al., 2015). The number of macrophages as well as dendritic cells increased in lymphoid tissue 3 days post infection. This increase maybe the result of antigen presenting role, cell debris phagocytosis role or indirect apoptotic role of macrophages especially since their presence was associated with severe lymphoid depletion (Ridpath et al., 2006).

Macrophages are an important innate immune cell in the pathogenesis of BVDV. Unlike monocyte, macrophages failed to produce BVDV new progeny after infection however the virus that can replicate and synthesize its viral proteins (Chase et al., 2015). BVDV infection doesn't affect the viability of MDM *in vitro* (Chase et al., 2004; Rajput et al., 2014). Interestingly, the macrophages and dendritic cells surrounding the apoptotic lymphocytes in the lymphoid tissue don't seem to be infected with BVDV (Liebleret al., 2003). *In vitro* infection of cultured macrophage with BVDV was associated with increased level of IL-1 and IL-6 but not TNF- α (Peterhans et al., 2003)

4.6.2. Bovine total lymphocyte population

Total peripheral blood lymphocyte populations include both specific T and B lymphocyte. T lymphocytes and its subpopulations is important for induction of cell mediated immune response against BVDV infected cells. While B lymphocytes are responsible for induction of humoral immune response against BVDV mediated via the production of neutralizing antibodies. Both humoral and cell mediated immune response

is very important for viral clearance from the animal post-infection. *In vitro* infection of peripheral blood lymphocytes with BVDV leads to induction of apoptosis in these cells based on the virulence of the infecting strain. Moreover, *in vivo* BVDV infection results in lymphoid depletion recoverable that may or may not be reversible based on the virulence of BVDV strain. (Ridpath et al., 2006; Chase, 2013).

4.6.3. Bovine lymphosarcoma cell line (BL-3)

Bl-3 is a non-adherent cell line derived from BL-3.1 cell line that was obtained from the American Type Culture Collection (ATCC, Manassas, VA). This BL-3.1 was originally derived from a Hereford calf suffering from B cell lymphosarcoma (Theilen et al., 1968). This cell line obtained from ATCC was infected with BVDV. The Bl-3 clone used in this study does not produce bovine leukemia virus (Harms and Splitter, 1992). The problem of BVDV contamination was solved using limiting dilution cloning to produce a BVDV-free BL-3 cell line. All clones were examined for BVDV and proven to be BVDV free by RT-PCR and immunohistochemistry following two blind passages of cell lysates on MDBK cells (Falkenberg et al., 2014)

5. Control and prophylactic measures for BVDV:

Bovine viral diarrhea virus has important characteristics including genetic diversity and a persistently infected that makes its control difficult in cattle populations. A systematic control program that utilizes diagnostic strategies to find and remove PI cattle, vaccination to increase fetal protection from infection, and biosecurity to reduce the risk of exposure to animals persistently or transiently infected with the virus is necessary for control of BVDV (Ridpath, 2013).

5.1. Control strategies regarding the risk of PI calves:

Because the PI animal is an important reservoir and transmitter of BVDV, control programs must first identify and remove these animals from the breeding herd. Exposing a breeding herd to PI cattle is likely to cause transient BVDV infections of susceptible cows and if pregnant, the subsequent vertical transmission of BVDV to their fetuses. To prevent contact with pregnant cows, PI animals should be removed prior to the start of the breeding season. To find and remove PI cattle prior to the start of the breeding season, all calves, all replacement heifers, all bulls, and all non-pregnant dams without calves (due to not becoming pregnant, aborting, or calf mortality) must be tested for PI status. Any female that is pregnant at the time the herd is tested should be isolated from the breeding herd and kept isolated until her calf is tested and found to be negative (Kelling et al., 2000).

Once a calf is identified as PI, it should be euthanized or removed for slaughter and the dam should be tested. Most dams of PI calves are not PI themselves, and if confirmed as non-PI, can re-enter the breeding herd because naturally acquired immunity is considered to be good protection from future fetal infections. Dams identified as a PI should be slaughtered immediately. (Orban et al., 1989).

In whole herd testing, IHC testing of skin samples is the test of choice because it can be accurately performed on animals of any age and a single sample is all that is usually needed. Other tests can be used for confirmatory testing for presumptive positive results from IHC when the history or clinical presentation does not concur with a diagnosis of PI. Using virus isolation or PCR to identify PI cattle requires a second test at

least three weeks following any positive samples to differentiate between transient viremia and PI with BVDV (Ridpath, 2013).

5.2. Vaccination as a prophylactic measure:

Application of a vaccination protocol is necessary to reduce the risk of fetal infection in the event of cowherd exposure to a viremic and shedding animal. Live, replicating vaccines (MLV) have inherent properties that may enable them to stimulate more complete protection against trans-placental infection. For that reason, one recommendation is to vaccinate unstressed, healthy heifers with MLV vaccine. Vaccine administration should be timed so that a protective immune response coincides with the first four months of gestation. This is done to maximize the potential for adequate immunity to protect against fetal infection and reproductive failure or the birth of PI calves. In heifers not previously vaccinated, the primary vaccine series should consist of two doses. The first dose should be given when the heifers are six months of age or older, and the second dose should be given two months before breeding. Beef cows should be revaccinated annually before breeding according to label directions (Kelling, 1996).

In vitro work has indicated that although there were large variations in the vaccine-induced virus neutralizing titers of individual colostrum-deprived calves vaccinated with two doses (21 days between doses) of an inactivated BVDV vaccine or a modified live, temperature sensitive BVDV vaccine, serum from each animal was capable of neutralizing a wide range of antigenically diverse European and American isolates of BVDV, including genotypes I and II (Hamers et al., 2001). Other work has shown that administration of a single dose of a modified live vaccine against BVDV stimulated an antibody response in seronegative cows that was detectable for at least 18

months. These antibodies were able to cross neutralize 12 antigenically diverse strains of BVDV (Makoschey, 2001).

Efficacy of maternal vaccination to provide fetal protection when the dams were experimentally challenged ranges from 25% to 100% for inactivated vaccines (Brownlie et al., 1995), and from 58% to 92% for modified live vaccines (Dean et al., 2003). Dams have measurable levels of anti-BVDV antibody following vaccination and fetal protection appears to be improved by vaccination, making a planned vaccination program important for BVDV control. However, a sufficient amount of virus is able to escape inactivation by circulating antibodies in some dams to cause transplacental infection, abortion, and the development of persistent fetal infection, making vaccination programs inadequate to control BVDV by themselves (Brownlie et al., 1998).

SUMMARY

BVDV is an immune suppressive viral agent that is associated with immune dysfunction in the infected animals. This immune dysfunction is mainly due to severe lymphoid depletion associated with high virulent strains of BVDV. The mechanism that explains this lymphoid depletion and the involvement of macrophages in such mechanism is still ambiguous. The aim of this study was to explore the role BVDV-infected MDM in lymphocyte apoptosis and to shed light on possible new mechanisms by which BVDV immune suppression can be explained.

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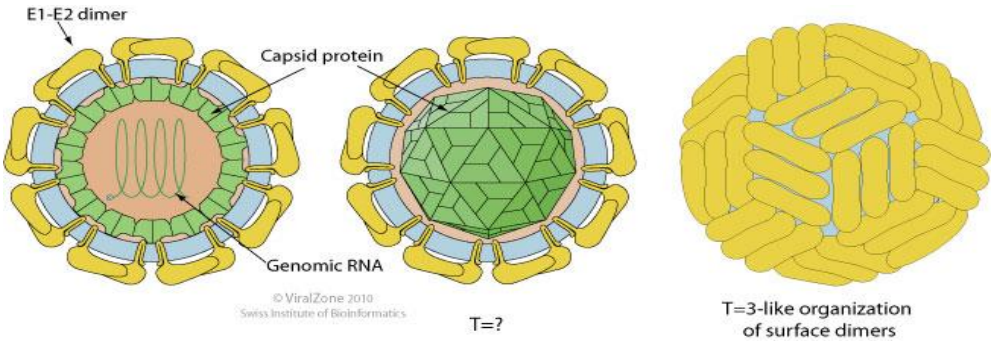

Figure 1.1. The Structure of BVDV. Adapted from ViralZone 2010, Swiss Institute of Bioinformatics http://viralzone.expasy.org/all_by_protein/39.html

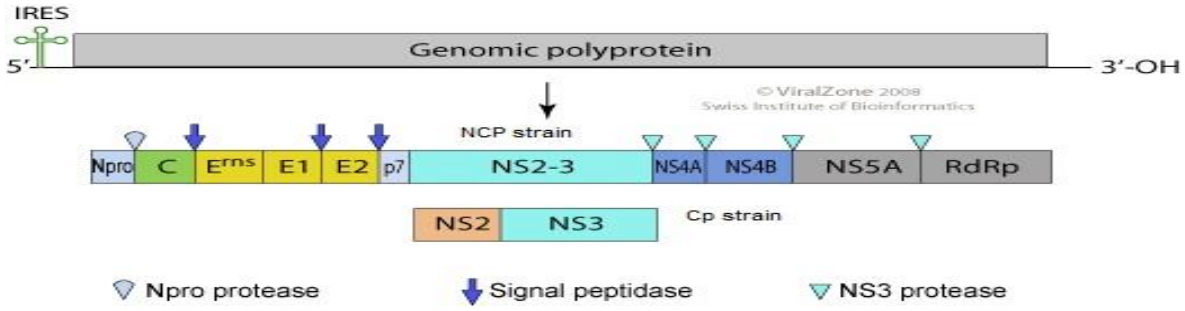

Figure 1.2. Schematic representation of BVDV encoded proteins. Adapted from ViralZone 2008, Swiss Institute of Bioinformatics.

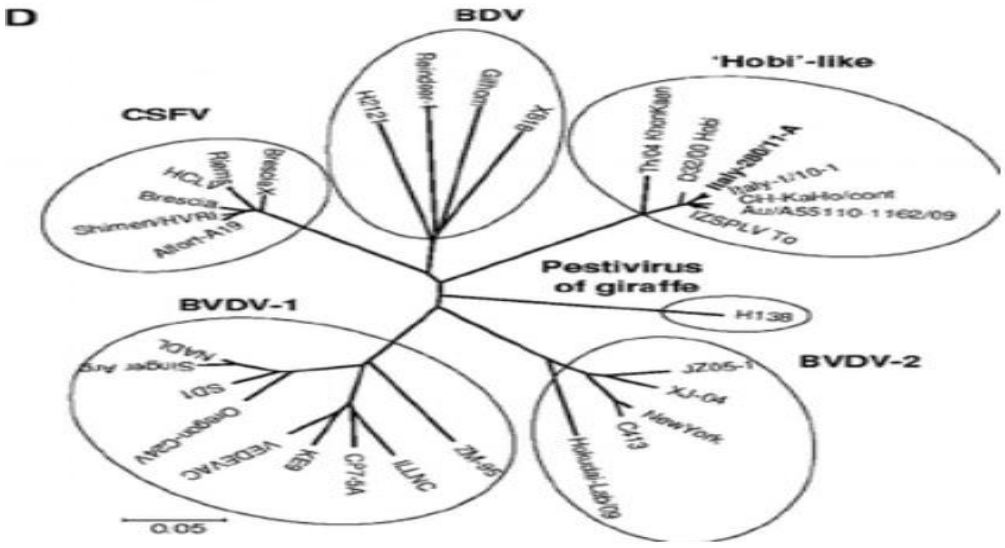

Figure 1.3. Phylogenetic tree showing the difference between the three genotypes of BVDV (Adapted from Decaro et al., 2012)

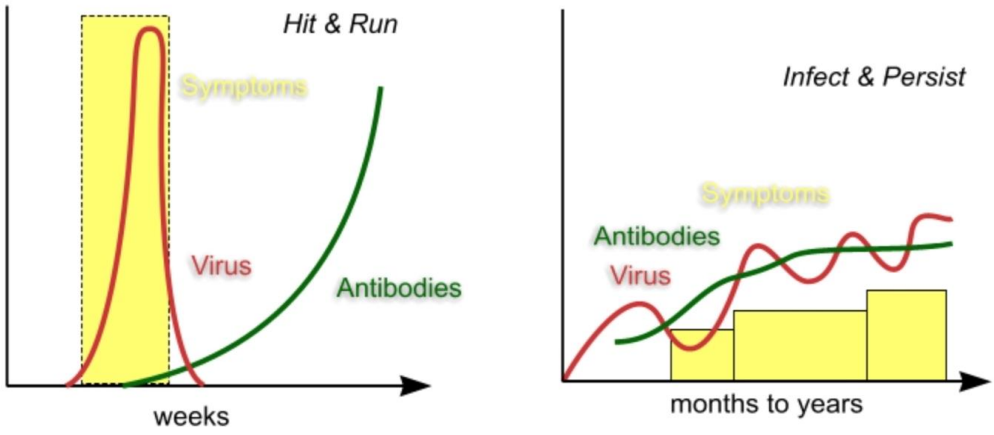

Figure 1.4. Strategies of BVDV infection. Adapted from Institute of Veterinary Virology, University of Bern http://www.bvd-info.ch/veterinarians/types_of_infection.html

Author	Country	Cattle Type	Reported Impact
Chi et. al *Prev Vet Med*	Canada	Dairy	$31.07 per cow
Duffell et. al *The Veterinary Record*	UK	Dairy	$31.10 to $88.75 per cow
Gunn et. al *The Veterinary Record*	Scotland	Beef cow-calf herds	$72.68 per cow per year
Hessman *BVD Control Conference*	US	Beef feedlot	$41.17 per head
Houe et. al *Vet. Microbiol.*	Denmark	Dairy	$20.00 to $57.00 per calving
Larson et. al *Bovine Practitioner*	US	Beef cow-calf	$15.33 to $20.16 per cow
Moennig et. al *Animal Health Res. Reviews*	UK, Norway, and Denmark	All breeds	$10.00 to $40.00 per calving
Ridpath *Hoard's Dairyman*	US	Dairy	$35.00 to $65.00 per calving

Table 1.1. The economic impact of BVDV. Adapted from ADM Animal Nutrition 2005 http://www.admsecondcrop.com/Beef/Beef%20Technical%20Bulletins/Beef%20BVDV%20Viral%20disease%20bovine%20viral%20diarrehea.htm

CHAPTER 2

THE INDIRECT EFFECT OF BVDV ON IMMUNE SUPPRESSION: THE ROLE OF INFECTED MACROPHAGES IN LYMPHOCYTE APOPTOSIS

ABSTRACT

BVDV is an important pathogen of cattle that affects both the dairy and beef industry causing severe economic losses. The main problem of BVDV is the immune suppression that is associated with lymphoid depletion shortly post infection. The mechanism of this immune suppression is not well understood. In this *in vitro* study, we sought to determine the indirect effect of BVDV infection in macrophages as a key player in the pathogenesis of BVD especially, the apoptotic effect induced on lymphocytes. We infected bovine monocyte-derived macrophages (MDM) with either high or low virulent strains of cytopathogenic or non-cytopathogenic strains of BVDV and collected supernatants that were used for treatment of the fresh peripheral blood lymphocytes or BL-3 B-cell line. We found that only supernatant from the high virulent BVDV strain induced lymphocyte apoptosis. Furthermore, direct infection of lymphocytes with the same virulent strain did not induce significant apoptosis. We investigated the apoptosis-related cytokine profile of the infected macrophages by qRT-PCR with different BVDV strains. There was no significant difference in the transcriptional profiles between strains so cytokine induction does not seem to be a mechanism of induced apoptosis. We then investigated the role of possible secreted viral proteins as the mechanism of indirect apoptosis of lymphocytes by treating the supernatants with specific antibodies against BVDV and its proteins. These treated supernatants still had lymphocyte apoptosis activity, ruling out their responsibility as well. Our data suggest an important role of

macrophages in the mechanism of lymphocyte depletion by high virulent strains of BVDV, however further studies are required to determine the identity and mechanism of action of these apoptotic factors present in the supernatant of the infected macrophages.

INTRODUCTION

BVDV causes immune dysfunction that affects cattle and other ruminant that disrupt the current vaccination-based control strategy and cause severe economic losses. The immune dysfunction associated with BVDV is multifactorial but one of the mechanisms is believed to be a consequence of lymphoid depletion that could be mild or severe based on the virulence of BVDV strains. The lymphoid depletion in some cases is so severe with some high virulent strains that animals never clinically recover and either die or are stunted (Ammari et al., 2012; Falkenberg et al., 2014; Liebler-Tenorio et al., 2003). The exact mechanism by which BVDV exerts its apoptotic effect is not well understood. Several studies suggest the possible role of macrophages in lymphoid depletion and immune dysfunction associated with BVDV. These studies demonstrated the importance of monocytes in lymphocyte apoptosis in a mixed population of monocyte and lymphocytes (Pedrera et al., 2009, Lambot et al., 1998). However, the role of apoptotic-associated cytokines, which are important as the mechanism of action for lymphocyte apoptosis for CSFV, does not appear to be important for BVDV (Pedrera et al., 2009). It has also been hypothesized that the pestivirus specific secreted E^{rns} glycoprotein (Fetzer et al., 2005) could play a role in the apoptosis mechanism associated with CSFV (Bruschke et al., 1997).

Only CP but not NCP strains can induce direct apoptosis to PBMCs (Zhang et al., 1996, Lambot et al., 1998). However most severe immune dysfunction due to BVDV infection is caused by non-cytopathogenic strains and associated with severe lymphoid depletion (Falkenberg et al., 2014). It has been proposed that NCP high virulent 1373 strain should be considered as lympho-cytopathogenic biotype since this virulent strain

doesn’t induce CPE on the susceptible cell lines but can induce lymphocyte apoptosis 3 days post infection (Ridpath et al., 2006).

To investigate the direct and indirect effect of different strains of BVDV on lymphocytes apoptosis, two types of lymphocytes were used. The first one was fresh lymphocytes from peripheral blood that included B, T and gamma-delta T specific lymphocytes in addition to NK cells. The second source of lymphocytes was BVDV-free BL-3 cell line that is a non-adherent B cells that is derived from BL-3.1 cell line obtained from the American Type Culture Collection (ATCC, Manassas, VA) (Falkenberg et al., 2014). The direct apoptotic effect of BVDV was examined by direct infection of lymphocytes with different BVDV strains. The indirect effect was investigated by infection of the MDM with three different strains of BVDV for 2 different time points followed by treatment of lymphocytes with the irradiated supernatant of infected macrophages (Elmowalid, 2003). Parallel to that the apoptosis-related cytokine profile of the infected MDM with different strains of BVDV has been studied.

MATERIALS AND METHODS

Animals

Six healthy Holstein Friesian calves (6-8 months of age) housed at Dairy Farm, South Dakota State University (SDSU), Brookings, SD, USA were used in this study. The SDSU Institutional Animal Care and Use Committee approved animal handling and blood collection. The animals were confirmed as BVDV-free by virus isolation followed by immunoperoxidase assay (IPA) (Fulton et al., 1997)

Virus

Three strains of BVDV were used in the current study: BVDV 2a high virulence 1373, BVDV 2a low virulence 28508 and 296C (Ridpath et al., 2016). These strains have been used before in examining the direct apoptotic effect of the virus. These strains were already prepared as lab strains and were preserved at -80 (Table 2.1.). All strains were titrated using TCID50 titration assay (Reed and Muench, 1938). Briefly, MDBK cells cultured in T 75 flask were trypsinized followed by culturing of the disaggregated cells into 96-well plate at a seeding density of $5x10^4$ cell suspended in180 µl MEM / well. The virus then diluted in 10-fold serial manner simply by adding 20 µl of the virus to each well in the first column followed by mixing with 180µ cell suspension and transferring that to the next column. The last 2 columns were kept uninfected as a negative control. The infected cells were incubated at 37°C in a humidified CO_2 incubator for next 3-4 days (Nuaire, NU 2700). For the CP strain, the plate was examined every day for CPE of the virus. The highest dilution showing CPE was used as an endpoint to calculate virus titer based on Reed and Muench equation (Reed and Muench, 1938) as follows:

$$TCID_{50}/ml = (PD - \log \text{dilution above } 50\%) \times 50$$

Whereas, PD= proportionate distance that can be calculated using the following equation: Proportionate distance (PD) = [(% CPE at dilution above 50%) – (50%)]/ [(% CPE at dilution above 50%)- (% CPE at dilution below 50%)]

For NCP BVDV, the endpoint was determined by immunoperoxidase assay (Fulton et al., 1997) with some modifications in the reagents used. Briefly, infected MDBK cells were stained with 15c5 E^{rns} specific monoclonal antibody (IDEXX Laboratories, Westbrook, ME, USA) followed by biotinylated rabbit anti-mouse IgG (Zymed, Invitrogen, Frederick, MD, USA), Streptavidin-HRP (Invitrogen, Camarillo, CA, USA) and AEC reagent (3 amino-9 ethyl-carbazole) (Sigma-Aldrich, St. Louis, MO, USA). The endpoint for NCP BVDV was determined by the presence of red stained cell showing BVDV protein.

Madin Darby bovine kidney (MDBK) cells

BVDV-free MDBK cells (passage 113-135) were grown in minimal essential medium (MEM, Gibco BRL, Grand Island, NY) (pH 7-7.4) supplemented with 10% BVDV-free fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT), penicillin (100 U /ml) and streptomycin (100 µg /ml). MDBK cells were maintained at 37°C with 5% CO_2 in humidified incubator and were used for viral titration and propagation.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated as previously described (Rajput et al., 2014) with modifications. Briefly, 120 ml of heparinized blood was collected by jugular venipuncture from 6-8 month heifers, using a 60 ml syringe containing 1 ml of 1000 U/ml heparin sulfate (Sigma, Sigma Chemicals, St. Louis, MO, USA). The heparinized blood was diluted 1:1 with PBS and overlaid on SepMate™

lymphoprep 50 ml tubes (Stemcell Technologies, Cambridge, MA, USA) gradients and centrifuged at 1200 Xg for 20 min at room temperature RT using a Beckman J6-MI centrifuge. After centrifugation, the plasma was removed by aspiration and the buffy-coat layer carefully transferred by quickly poured into a clean 50 ml conical tube (Falcon, Oxnard, CA, USA). The buffy coat was washed 5 times with PBS followed by centrifugation at 120 Xg for 10 min at RT. The viability of PBMCs was determined by trypan blue exclusion assay using 0.4% trypan blue stain (Strober, 2001). The following formula was used to calculate the cell viability of the monocytes:

Cell viability % = Number of viable cells (non-trypan blue stained cells-trypan blue stained cells) X 100/Total counted cells.

The PBMC pellet was re suspended in RPMI 1640 medium (GE Healthcare, Hyclone Laboratories, Logan, UT, USA) supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100µg/ml) to achieve a final concentration of $1X10^6$ cells/ ml. The cells were incubated in T_{175} Flask for 3 h at 37˚C (Corning, NY, USA).

Plastic adherence based monocyte separation and morphological identification

The monocytes were isolated by the plastic adhesion method (Mwangi et al., 2005, Rajput, 2014) with modifications. Following incubation for 3 h at 37˚C, the floating cells were discarded and flasks were washed 5 times with PBS how much?? and the wash discarded. The adherent cells were detached with Accutase™ (eBioscience, San Diego, CA, USA). Detached cells were washed two times by suspending them in PBS and pelleting by centrifugation at 120 Xg for 10 min at RT. The detached cells were identified as monocytes with a characteristic kidney shape nucleus by H&E stain. Briefly, 1 ml of the detached cells was concentrated on a Cytospin™ slide (Thermo Shandon,

USA). The slide with the concentrated cells could dry for 15 min in the biosafety hood then stained for 15 min using Mayer's hematoxylin solution (Sigma-Aldrich, St. Louis, MO, USA). The slide was rinsed with tap water and re stained with alcoholic solution of eosin for 40 seconds (Sigma-Aldrich, St. Louis, MO, USA). Finally, the slide was double rinsed with 96-100% ethanol?? Or methanol alcohol and the slide was examined under oil emersion lens using an inverted compound microscope (Olympus, PA, USA). Monocytes were counted 3 times and the average was taken. The purity of monocytes was calculated as follow:

Monocyte % = Number of monocytes (kidney shape nucleus) X 100/Total counted cells.

Peripheral blood monocytes-derived macrophages (MDM) culture and characterization

MDM were cultured as described by Elmowalid, 2003 with modifications. Briefly, the adherent monocytes were cultured in RPMI 1640 (GE Healthcare, Hyclone Laboratories, Logan, UT, USA) supplemented with 10 % FBS, penicillin (100 U/ml) and streptomycin (100μg/ml) at a final concentration of $1x\ 10^5$ cells/ well in 48-well plate followed by incubation for 7 days at 37˚C in CO_2 incubator. The incubated cells were fed every other day simply by replacing half of the conditioned media with fresh complete RPMI. At the 7^{th} day, the MDM were characterized phenotypically as MHCI, MHCII, CD11b and CD14 positive for at least 84% of the cells. This was done as described by Rajput, 2013. Briefly, MDM were detached using Accutase™ (eBioscience, San Diego, CA, USA) followed by centrifugation for 10 min at 1000 Xg and washing with PBS. The number of the cells was adjusted to $1x10^6$/ml. Four primary mouse mAb antibodies for MHC-I (H58A), MHC-II (H42A), CD11b (MM10A) and CD14 (MM61A) (Monoclonal Antibody Center, WSU, Pullman, WA, USA) were used to characterize the cells. Cells

were incubated with 50µl of diluted primary antibodies 1:100 in PBS containing 1% FBS at 4°C for 10 min followed by washing with PBS. This was followed by staining with 1:1000 in PBS diluted FITC labeled anti-mouse secondary antibody (VMRD Inc., Pullman, WA, USA, containing 1% FBS, at 4°C for 10 min. The stained cells were washed two times with PBS and were fixed with 200µl of 1% paraformaldehyde. The percentage of cells expressing surface markers was calculated using BD Accuri™ C6 Plus Flow Cytometer (BD Biosciences, CA, USA).

BVDV infection of MDM

MDM was infected with 3 different strains of BVDV, 1373, 28508 and 296C in triplicates as described by Elmowalid, 2003 with modifications. Briefly, the viral strains were adjusted to infect MDM at multiplicity of infection (MOI) of 1 by diluting the virus stock with plain RPMI 1640 medium. Seven-day old MDM washed with 1x PBS to remove old medium containing floating dead cells then infected with diluted virus strain with $1X10^5$ TCID50 at MOI of 1 in a final volume of 100 µl. The infected cells were incubated for 1 h then washed again to remove the excess unbound virus and 500 µl of complete RPMI 1640 medium was added to each well. At least one column of the plate was mock infected with complete RPMI 1640 medium as a negative control. The infected MDM were incubated for 12, 24 or 48 h at 37°C in CO_2 incubator.

Direct immunofluorescence assay

MDM infection with BVDV was monitored by direct immunofluorescence using FITC-labeled BVDV-specific polyclonal antibody of swine origin (VMRD Inc., Pullman, WA, USA) to confirm virus infection of MDM. The direct immunofluorescence was done using the SOP provided by (ADRDL, SDSU, USA). Briefly, the infected MDM

were air-dried then fixed by adding 100 µl of 20% acetone diluted in 1x PBS and incubated at RT for 10 min. The acetone was discarded and 50 µl of FITC conjugated BVDV-specific polyclonal antibody was added to each infected well as well as the mock infected negative control that work to exclude nonspecific fluorescence signal, this in addition to including un stained control by adding 50 µl of PBS. The whole plate was incubated at 37°C for 30 min and the plate was examined using a fluorescent inverted microscope (Olympus, PA, USA) (Figure 2.3).

Supernatant collection and irradiation

Supernatant collection and treatment was done as described by Elmowalid, 2003 with modifications. Briefly, the infected macrophage supernatants of the three BVDV strains were collected at 12, 24 and 48 h pi in 15 ml conical tubes. The supernatant was centrifuged at 1000 Xg for 10 min at RT to remove cellular debris. After dispensing each strain supernatant in 100x15 mm polystyrene sterile square petri dishes (Falcon, Oxnard, CA, USA), the supernatant was treated by UV irradiation at 15 cm for 20 min on ice to inactivate virus (Perler et al., 2000; Jungmann et al., 2001). The absence of any infectious viral particles in the treated supernatants was confirmed by IP assay and the result was contrasted to a positive 1373 infected MDBK. Finally, three different time point; 12, 24 and 48h UV-irradiated-supernatants for each strain of BVDV were used either directly for treatment of lymphocytes or aliquot and stored at -20°C (Figure 2.4).

Quantification of apoptosis-related cytokines by qRT-PCR of BVDV-infected MDM

RNA was harvested using the methods described by Rajput, 2013 with modifications. Briefly, the MDM infected with one of the 3 different strains of BVDV were collected at 2 different time points, 24 or 48 h pi, then washed 2 times with 1x PBS. The cells were

lysed by freezing at -80°C for 15 minutes and thawing at 4°C for 30 minutes. The washed, infected MDM were lysed and nucleic acid extraction done using RNeasy extraction kit (Qiagen, Valencia, CA, USA). The washed cells were kept at -80˚C if they were not extracted directly. A negative control using RNase, DNase free water (Invitrogen, Life Technologies, Thermo Fisher Scientific, PA, USA) was included to exclude any possible nucleic acid contamination every time NA extraction conducted. The extracted NA of different samples was normalized to 5 ng/µl using Nanodrop ND-1000 Spectrophotometer (Fisher Scientific, NH, USA). Around 2 µl of the normalized NA was used to start relative quantification of apoptosis-related cytokines; TNF-a, IL-1a, IL-1b and IL-6 for each sample in duplicates for each animal used in this study by using quantitative reverse transcriptase PCR (qRT-PCR). MDM from at least 3 different animals were used for infection with the one of three different strains of BVDV. qRT-PCR was done in 25µl reaction using Power SYBR® Green RNA-to-Ct™ 1-Step Kit (Thermo Fisher Scientific,, PA, USA). The quantification of cellular mRNA was done using an ABI 7900HT High-Throughput Real-Time Thermocycler (Applied Biosystems, Life Technologies, Thermo Fisher Scientific, PA, USA). The relative expression of mRNA was standardized using beta actin as a housekeeping gene to ensure the PCR efficiency via stable expression of the gene. Nucleic acid extracted from mock-infected MDM with complete RPMI 1640 medium was used as a negative control. Nucleic acid extracted from MDM treated with Concanavalin A (ConA) (Sigma-Aldrich, St. Louis, MO, USA) was used as a positive control. ConA-treated macrophages up-regulate the 4 cytokines used in this study. The cytokine primers used were described by Konnai et al., 2003 (Table 2.2). The qRT-PCR results were expressed as cycle threshold (CT) values;

the CT value is the number of PCR cycles that is required to obtain the threshold detection. Each qPCR experiment was followed by heat dissociation curve step to verify the result and to exclude nonspecific amplification. qRT-PCR results were analyzed using relative expression software tool (REST©2009 software) (Pfaffl et al., 2002).

Fresh lymphocyte culture

The PBMCs were isolated as described above and monocytes removed by adhesion to plastic for 3 h at 37°C in CO_2 incubator. The non adhesive cells were collected and washed 3 times by PBS and centrifuged at 120 Xg for 10 min at RT to remove the platelets. The pelleted lymphocytes were suspended in RPMI 1640 medium supplemented with 15% FBS, penicillin (100 U/ml) and streptomycin (100µg/ml) to achieve final concentration of 1x 10^5 cells/ ml followed by culturing in 24-well plate with a seeding density of 1 ml/well. The plate then incubated at 37°C till either direct infection with BVDV strains or treatment with infected macrophage supernatant.

Examining the direct, indirect and the combined effect of BVDV strains on peripheral blood lymphocyte population

To examine the indirect effect of BVDV strains, peripheral blood lymphocytes were treated with 12, 24 or 48 h irradiated supernatants from BVDV-infected macrophages at a dilution of 1:1 and incubated for 12, 24 and 48 h post treatment as described by Elmowalid, 2003.

To examine the direct effect of BVDV strains, peripheral blood lymphocytes were infected with 1373 and 296C strains as described by Ridpath et al., 2007 with modifications. Briefly, the viruses were diluted using RPMI 1640 medium and adjusted to infect the lymphocytes at MOI of 1. This is followed by incubation for 1 h then the

plate was spun down for 10 min at 400 Xg and the excess virus was discarded. This was followed by re suspension of the infected lymphocyte pellet in complete RPMI medium, so that each well contain 1 ml of cell suspension, then incubation for 12, 24 and 48 h post infection.

To investigate whether the indirect effect had a synergistic effect with the direct effect on the lymphocyte apoptosis, we studied the combined effect of both direct and indirect apoptotic effect of 296C strain of BVDV. Briefly, 2 parts of the infected MDM 48h supernatant of 296C strain was added to one part of the diluted virus (MOI:1) and one part of $1x10^5$ lymphocytes suspended in 100µl of complete RPMI 1640 medium. Virus-lymphocyte mixture was incubated for 24 h at 37˚C in CO_2 incubator. We included also the direct effect alone as well as the indirect effect to compare between the 3 different effects: direct, indirect and the combined. Mock-infected controls were also included to exclude virus non-specific apoptosis simply by suspending the lymphocytes with complete RPMI 1640 medium.

BL-3 culture and infection

The BL-3 cell line was cultured and handled as described by Ridpath et al., 2006 with modifications. Briefly, the BVDV-free BL-3 cells were obtained kindly from Dr. Shollie Falkenberg and were propagated in suspension culture in RPMI 1640 medium (GE Healthcare, Hyclone Laboratories, Logan, UT, USA) supplemented with 15%FBS, penicillin (100 U/ml) and streptomycin (100µg/ml) to achieve final concentration of 1x 10^6 cells/ ml. T-25 flasks were used for incubation with each flask containing 30 ml cell suspension and incubated in an upright position in CO_2 incubator for 3 days at 37°C. The cells were sub-cultured using the protocol of Ridpath et al., 2006, by removing 2 parts of

the cell suspension and replacing with fresh complete RPMI 1640. The BL-3 cells were then cultured in 24-well plates (Falcon, Oxnard, CA, USA) at a seeding density of $1x10^5$ cells/ well. BL-3 cells were then treated indirectly or infected directly as described above for fresh lymphocytes.

Virus neutralization

Each viral strain was diluted to MOI of 1 and incubated at 37°C for 1 h with either E^{rns} specific 15c5 monoclonal antibody, 1mg/ml (IDEXX Laboratories, Westbrook, ME, USA) or with BVDV-specific polyclonal antibody (Kindly supplied by Dr. Fulton, Food Animal Research, Oklahoma State University) in a parallel experiment. The polyclonal antibody was previously examined for its neutralization effect against each of the 3 BVDV strains used and it was higher than >1:512 (data not shown). The BL-3 cell line was seeded at $1x10^5$ and allowed to be incubated with the virus-antibody mixture at 37˚C for 36 h post treatment in CO_2 incubator.

Supernatant neutralization

Twenty-four hour (24h) or 48h supernatants from the BVDV-infected macrophages with one of the three different strains was incubated at 37°C for 1h with E^{rns} specific 15c5 monoclonal antibody (IDEXX Laboratories, Westbrook, ME, USA) or with BVDV-polyclonal antibody (Kindly supplied by Dr. Robert Fulton, Food Animal Research, Oklahoma State University) in a parallel experiment. The mixture of the infected supernatant and antibody was prepared as 2 parts of infected supernatant incubated with one part of concentrated antibody. Three (3) parts of the supernatant/antibody mixture was added to one part of BL-3 cell line seeded at $1x10^5$ cells/well, followed by incubation at 37˚C for 36 h post treatment.

Apoptosis analysis using Annexin V staining

This was done using the AnnexinV staining kit (eBioscience, San Diego, CA, USA). Briefly the infected or supernatant treated lymphocytes was washed once with 1X PBS and the cells were spun down at 1000 Xg for 10 min and re suspended in 100µl of 1X binding buffer supplied by the kit as 4X stock solution. Five (5) µl of FITC conjugated annexin v antibody added to the lymphocytes suspended in 100µl of binding buffer and incubated for 10 min in dark. This was followed by washing the cells twice by suspending the cells in 1X binding buffer followed by centrifugation of the cells at 1000 Xg for 10 min. Five (5) µl of propidium iodide was added to the washed cells and the cells suspending in 1X binding buffer followed by centrifugation of the cells at 1000 Xg for 10 min. Finally, the washed cells were re suspended in 200µl of 1X BD FACS™ lysing solution followed by analysis with BD Accuri™ C6 Plus Flow Cytometer (BD Biosciences, CA, USA). The result was expressed as % of apoptosis

Statistical analysis

Data was analyzed using a student's t-test (Microsoft EXCEL, MAC 2011) to assess the significance of the differences between mean values of treated and control samples at each time point. Differences were considered significant at $P < 0.05$. Every experiment was achieved using at least 3 animals (replicates) and 3 repeats (performed 3 times for each replicate animal) to confirm the reproducibility of the methods used. The variations in results were calculated by standard deviation at each time point. For cytokine analysis, REST © 2009 that is based on analysis of variance (AVOVA).

RESULTS

Monocyte identification and MDM characterization

H&E staining of cells isolated using adherence as positive selection revealed characteristic mononuclear cells with kidney shaped nucleus with morphological characteristics of bovine monocytes with a purity of 88%±4 (Figure 2.1). Following maturation, the MDM purity ranged from 84 to 96%±2.6 by flow analysis using macrophage-specific surface markers. Flow analysis indicated MDM expression of 98.57 % MHCI, 97.25 % MHCII, 84.34% CD11b and 90.24% CD14 (Figure 2.2).

BVDV infection of MDM

The infected macrophages had BVDV-specific intra-cytoplasmic staining by direct immunofluorescence at12 h post infection for all 3 strains (Figure 2.3A, B, C). The mock infected negative control had no BVDV marked cytoplasmic fluorescent signal (Figure 2.3D).

Inactivation of infected supernatant

The infected MDM supernatant was UV irradiated to inactivate infectious BVDV particles to exclude direct effect of the virus. To check for the success of irradiation, MDBK cells were treated with irradiated supernatant and this was followed by immunoperoxidase (IP) assay to detect virus replication. The results of IP assay with UV-irradiated virus showed a complete absence of IP staining (Figure 2.4A) compared to the positive IP staining of 1373-infected-MDBK control (Figure 2.4B).

Role of cytokines in the indirect lymphocyte apoptosis

Quantitative RT-PCR for the infected MDM lysate at 3 different time points; 12, 24 and 48 h post infection was performed. The apoptosis-related cytokines didn't show

significant change at any of the three time points compared to negative mock-infected control. The positive Con A treated MDM lysate showed significant up-regulation comparing to negative mock-infected control (Figure 2.5.)

Indirect lymphocyte apoptosis and flow analysis

Three time-point supernatants from 12, 24 and 48h BVDV-infected macrophages with 3 different strains of BVDV were used to treat fresh peripheral blood lymphocytes or BL-3 cell line. The 12 h supernatants of all strains didn't show any significant apoptosis from the mock-infected control (Figure 2.6A). Both 24 and 48 h supernatants of both high virulent 1373 and virulent 296C resulted in apoptotic effect on the both fresh and BL-3 lymphocytes ($p<0.05$) (Figures 2.6B, C; 2.7A, B C). All supernatants from 12 thru 48h of the low virulent NCP 28508 didn't show any significant apoptotic change from the mock infected control for either BL-3 lymphocytes or peripheral blood total lymphocyte population (Figure 2.6B, C)

Direct lymphocyte apoptosis and flow analysis

The direct infection of lymphocytes either from peripheral blood or BL-3 cell line varied based on the biotype of BVDV (Figure 2.8). CP 296c led to a marked and significant apoptosis ($p<0.01$) from 12 h pi of 19±4.2% which increased to 44±6.5% through 48 h pi compared to 7.5% and 18% at 12 and 48 respectively for the mock-infected controls. However, both NCP high virulent 1373 and low virulent 28508 strains didn't induce significant changes ($p<0.05$) compared to the mock-infected control until 48 h post infection (Figure 2.8.). A comparison between the direct and indirect effect of high virulent 1373 strain was conducted and the indirect effect at 24 and 48h supernatant was

2-3 times greater than the direct effect (Figure 2.9). The direct effect was not significantly different from the mock-infected control (Figure 2.9)

The combined effect of 296C and its infected MDM supernatant on lymphocyte apoptosis

296C induced a marked direct as well as indirect apoptotic effect on lymphocyte population Figure 2.10). To investigate whether these 2 effects could be synergistic or antagonistic, the BL-3 lymphocytes were treated with supernatant of infected MDM with 296C along with direct infection with the virus in the same well. The results showed that there was no significant difference in the percentage of lymphocyte apoptosis induced by the direct effect alone compared to the combined effect (Figure 2.10). However, the combined effect induced more lymphocyte apoptosis but not significantly different ($p = 0.055$) than the indirect effect of 296C alone ($p<0.05$) (Figure 2.10).

Role of E^{rns} glycoprotein in the indirect lymphocyte apoptosis

To investigate which viral factors were responsible for the indirect lymphocyte apoptosis, 15c5 E^{rns} specific mAb was used to neutralize the supernatant of infected MDM of the CP 296C or NCP 1373 strain of BVDV. Following neutralization, the BL-3 cells were treated with the neutralized infected supernatant of one of these 2 strains and the percentage of lymphocyte apoptosis was calculated by FACS analysis. There was no significant difference in the percentage of lymphocyte apoptosis between that infected supernatant treated with mAb and the untreated for both strains. The mAb-treated or untreated 24 and 48h supernatant induced significant lymphocyte apoptosis ($p<0.05$) that ranged from 15.35±5.62 to 18.1±4.33% for 24h supernatant and from 27±1.4 to 29±3.7% for 48h supernatant. They were all significantly different compared to the mock-infected

cells that only induced around 5.45±3.23 to16±2.3% at 36 h post treatment (Fig 2.11A & B).

Role of other viral proteins in lymphocyte apoptosis

The role that any other viral proteins could plat in the indirect lymphocyte apoptosis was determined using apoptosis neutralization test. Infected MDM supernatant of either 1373 or 296C was further treated with BVDV-specific polyclonal antibody and the apoptotic effect was measured by FACS analysis. Like E^{rns} mAb, BVDV polyclonal antibody failed to protect against apoptosis in BL-3 cells (Fig 2.11A & B). Polyclonal antibody-treated supernatant induced similar levels of apoptosis as that induced by supernatant alone with either BVDV strains. The percentage of apoptosis was almost the same as that obtained from mAb data (Fig 2.11A & B).

DISCUSSION

This study sheds light on the role of macrophages in the process of lymphoid apoptosis associated with BVDV infection. Highly virulent BVDV strains can induce both direct and indirect apoptosis on lymphocyte populations regardless of the biotype the strains. These results differ from the results reported by Zhang and his group that only CP BVDV strains can induce apoptosis (Zhang et al., 2016). This difference is probably due to the usage of different strains. Our results are consistent with the results of Pedrera et al., 2009 that did not relate the apoptotic effect to the biotype of the strain. Our results support the results of Ridpath et al., 2006 that NCP high virulent 1373 strain can induce lymphocyte cytopathogenesis (apoptosis). This third biotype, lympho-cytopathogenic is unique since cytopathology is only induced in lymphocytes while 1373 remains NCP on susceptible non-lymphoid cell lines (Ridpath et al., 2006).

In the current study, we found that at least one virulence factor of 1373 BVDV strain is probably associated with the severe lymphoid depletion as low virulent 28508 failed to induce apoptosis on the fresh lymphocytes. Our results agree with the association between virulence and the degree of immune suppression that is associated with lymphoid depletion *in vivo* (Ammari et al., 2012, Falkenberg 2014).

The role of macrophages in lymphocyte apoptosis was investigated in this study. Our results suggested that the supernatant of infected macrophages with only virulent BVDV strains induced lymphocyte apoptosis. These results provide an explanation to the observation that there are increased number of macrophages 3 days post infection prior to lymphoid depletion (Pedrera et al., 2009). Moreover, this indirect effect mediated by macrophages wasn't significant until 24 h post infection with virulent BVDV strains,

suggesting limiting the infection would decrease or prevent the severe immune suppression induced by BVDV.

In contrast, the CP strain of BVDV was capable of induction of a fast and direct apoptotic effect on lymphocytes comparing to the NCP virulent strain. The NCP BVDV didn't exert any direct apoptotic effect until as late as 3 days' post infection which is consistent with the late apoptotic effect of 1373 as a lympho-cytopathogenic biotype of BVDV (Ridpath et al., 2006). Interestingly, we noticed that high virulent 1373 strain of BVDV induced more marked and faster apoptotic effect on indirectly as compared to the direct effect. These results suggest that the severe and faster immune suppression associated with high virulent 1373 is likely due to the indirect effect mediated by macrophages.

Cytokines associated with apoptosis produced by BVDV-infected macrophages do not appear to be involved in lymphocyte apoptosis. There was no significant change in TNF- a, IL-1a, IL-1b or IL-6 mRNA levels. These results were different from those described for CSFV as CSFV is capable of induction of both direct and indirect lymphocyte apoptosis and TNF-a is involved in this process (Choi et al., 2004). Although TNF induction appears to be an apoptotic mechanism for CSFV, this does not appear to be true for BVDV. This lack of involvement of TNF-a is in agreement with previous results that ruled out a role for TNF-a in BVDV lymphocyte apoptosis (Pedrera et al., 2009).

Previously, it has been reported that E^{rns} glycoprotein plays an important role in the process of lymphocyte apoptosis induced by BVDV and CSFV (Bruschke et al., 1997). However, the use of E^{rns} specific monoclonal antibody with the infected

macrophage supernatant in this study revealed that E^{rns} glycoprotein doesn't play a significant role in the indirect lymphocyte apoptosis. This difference may because they used a cloned BVDV with E^{rns} glycoprotein of CSFV but not the E^{rns} of BVDV. We have also ruled out the effect of other viral proteins on lymphocyte apoptosis using BVDV-specific polyclonal antibodies. The polyclonal treated supernatant failed to protect against indirect lymphocyte apoptosis. However, there was a protective effect exerted by both E^{rns} specific monoclonal and BVDV-specific polyclonal antibodies against the direct apoptotic effect. This was likely due to blocking effect of these antibodies which indicate they neutralize virus efficiently but the macrophage produced lymphocyte apoptosis factor is probably not of viral origin.

The current work also has revealed that CP virulent 296 strain of BVDV can induce both direct and indirect apoptotic effect on lymphocytes. This is in partial agreement with previous work that proposed that lymphocyte apoptosis is associated with the direct effect of the virus on the infected cell rather the interaction between infected and normal cell (Zhang et al., 1996, Lambot et al., 1998). However, in a parallel experiment, we have found that the sum of direct and indirect apoptotic effect of 296C isn’t significantly different from the direct effect alone that probably indicate that the indirect apoptotic effect of CP virulent strain of BVDV is not exaggerating the overall apoptosis induced by that strain. Moreover, we have also noticed that while the additive effect of both direct and indirect wasn't significant compared to the direct effect alone, the direct effect was significant compared to the indirect effect. This data suggested that CP virulent BVDV exerted its apoptotic effect on lymphocytes mainly via the direct effect of the virus.

The use of BL-3 cell line in this study was also useful as it gave similar results as compared to peripheral blood lymphocytes. This finding with BL-3 cells was consistent with what was reported previously of the apoptotic effect of high virulent BVDV strains on the BL-3 cell line (Ridpath et al., 2016). The degree of apoptosis induced in the lymphocytes of both peripheral blood or BL-3 cell line was almost the same. This finding causes us to speculate that B cells are more likely to be affected than T lymphocytes but this hypothesis needs to be tested.

GENERAL CONCLUSION

BVDV is considered the cattle industry's most costly endemic viral diseases. As a potent immunosuppressive agent, BVDV infection can lead to vaccination failure and makes a whole farm susceptible to infection with other bovine pathogens. It was important for our study to focus on immune dysfunction caused by BVDV through apoptosis of specific lymphocytes that are important for clearance of the virus from the body of infected animals. Our data suggested that the immune dysfunction associated with high-virulent NCP 1373 strain was mainly due to the indirect effect mediated by macrophages. Unlike high-virulent 1373, cytopathogenic BVDV strain 296C lymphoid depletion was due to both direct and indirect effects, but the direct effect seems to be more important. Unlike CSFV, neither TNF or E^{rns} glycoprotein induce BVDV lymphocyte apoptosis. This led us to hypothesis that the indirect lymphocyte apoptotic effect of was likely mediated by host but not virus factors. Further we found that other apoptosis-related cytokines that play an important role in the indirect apoptosis didn't seem to play a role in induction of indirect BVDV lymphocyte apoptosis.

Further investigation of the factors present in the supernatant of the infected macrophages with high virulent strains need to be conducted so that we have better understanding of the disease mechanism. This can lead to finding a way to block these factors that can be used as a therapeutic practice in the acutely infected animals to help improve recovery. Understanding these factors better would also help improve the current control strategies and decrease the risk and the economic losses of BVDV.

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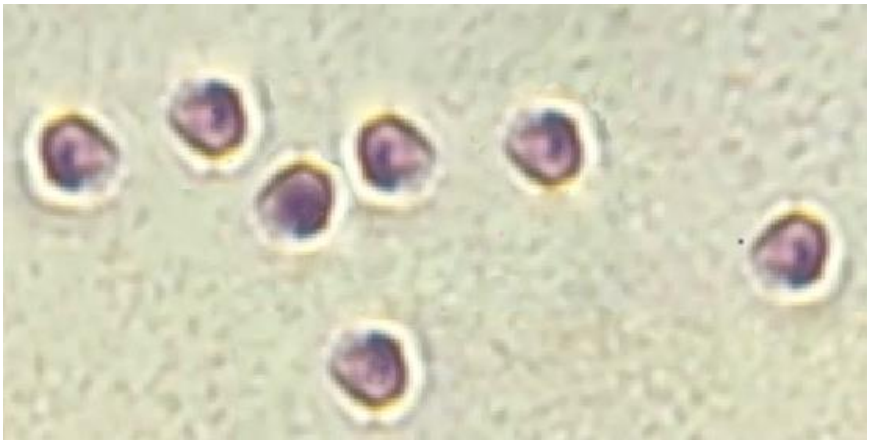

Fig 2.1. Isolated bovine monocytes: Bovine monocytes stained with H&E showing kidney shape nucleus with 88±4.2% purity.

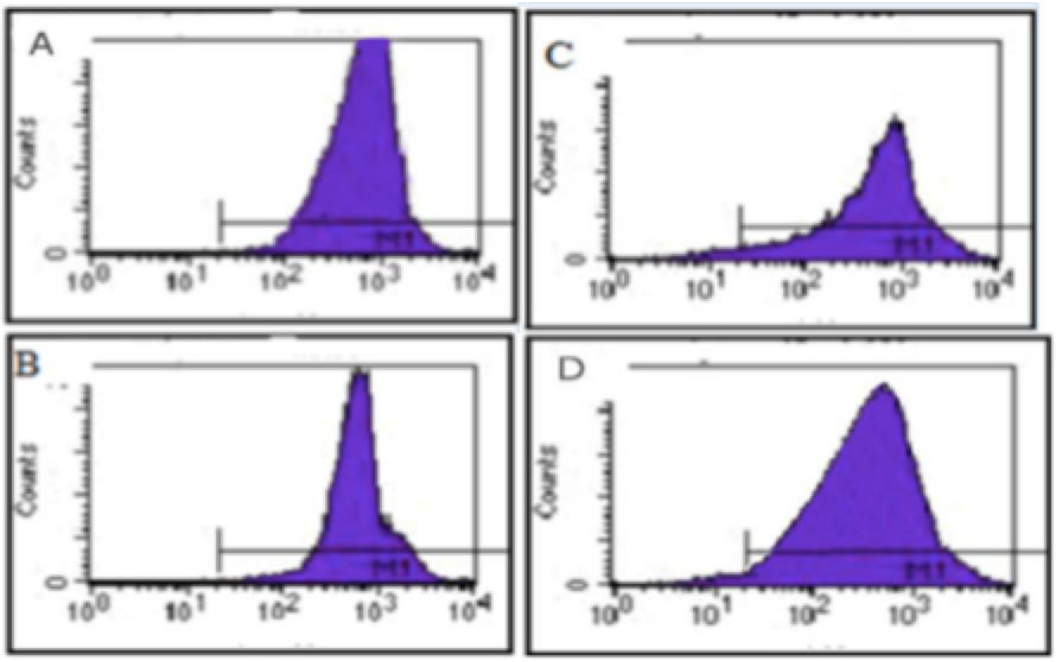

Fig 2.2. Cultured bovine MDM: MDM expressing MHC I (98.57±0.73) (A), MHC II (97.25±2.81) (B), CD11 b (84.34±3.89) (C), and CD14 (90.24±5.99) (D)

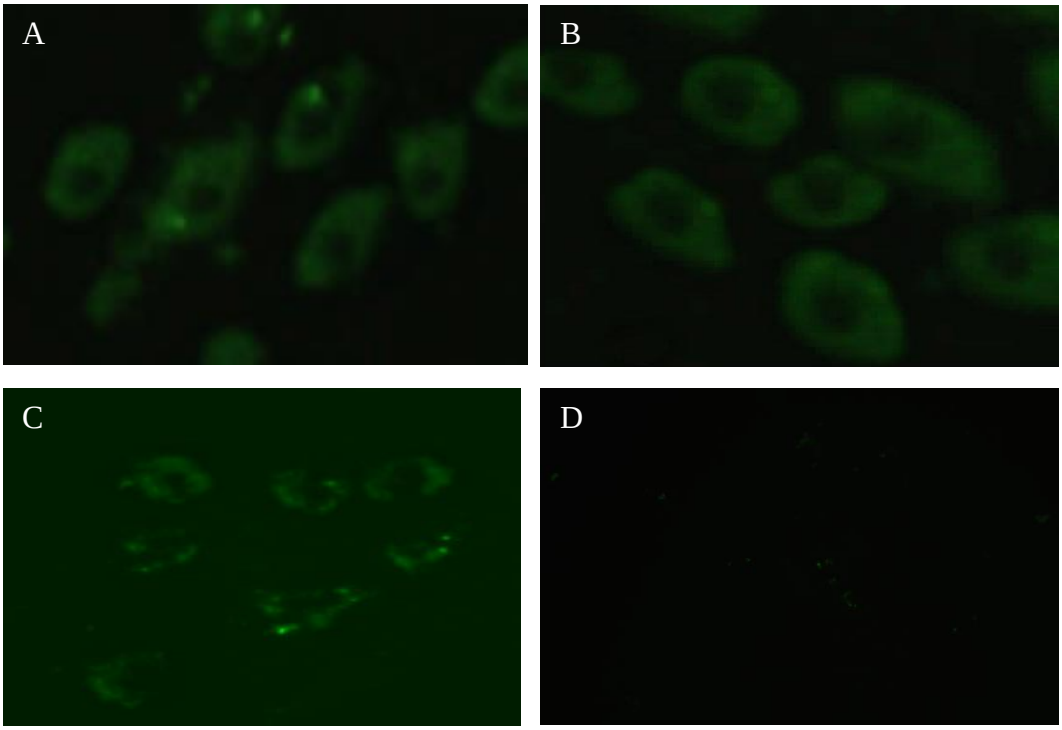

Fig 2.3. BVDV infection of MDM: Direct immunoflourescence assay showing BVDV infection of MDM and intra-cytoplasmic replication of 1373 (A), 28508 (B), 296 C (C) and mock infected (D)

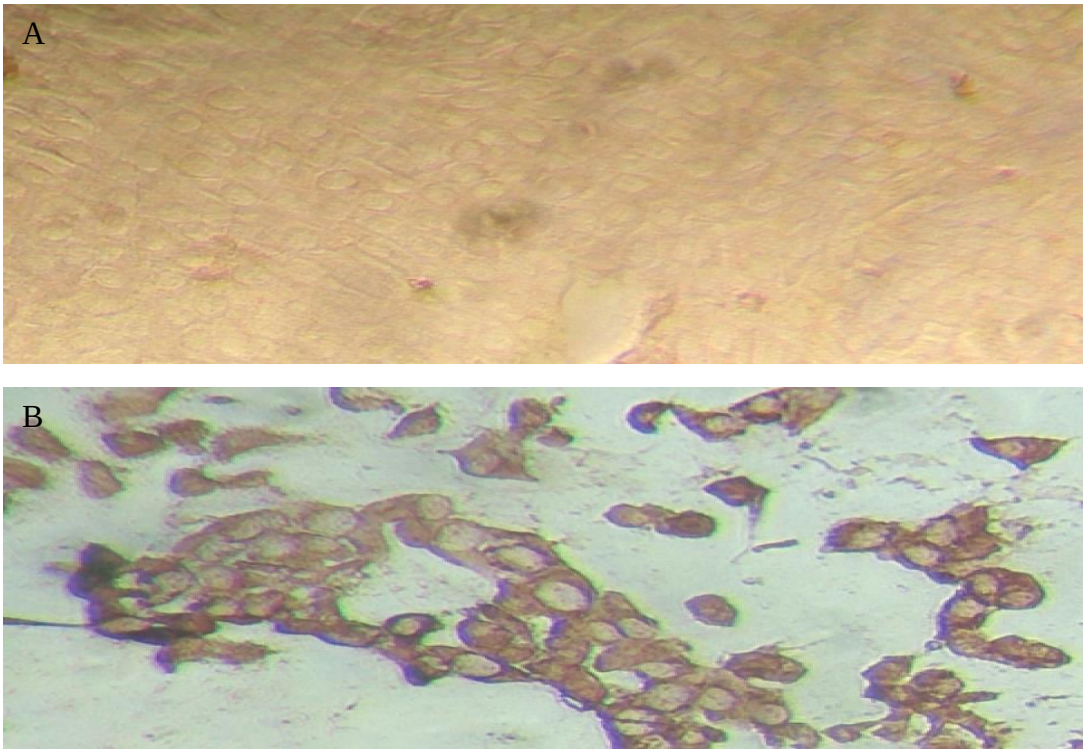

Fig 2.4. Successful irradiation of MDM infected sup: Immunoperoxidase assay showing inactivation of 1373 infected MDM supernatant (A) comparing to positive 1373 control intra-cytoplasmic replication

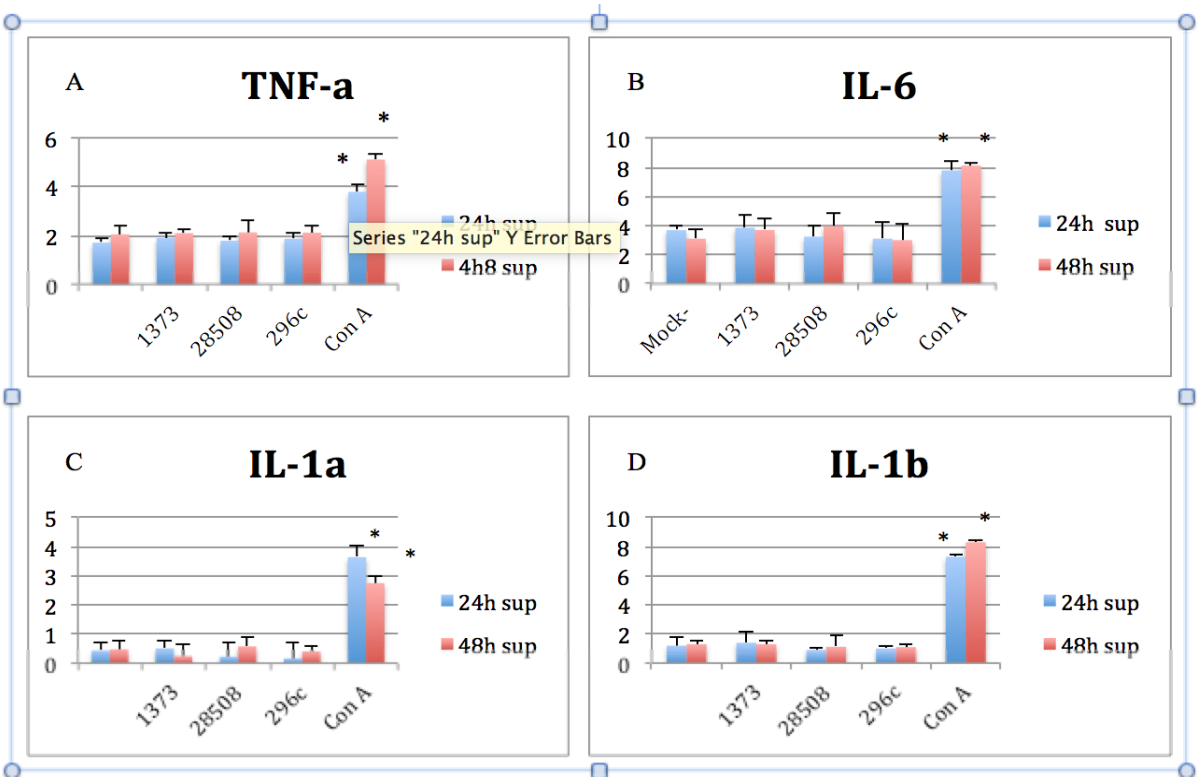

Fig 2.5. Quantification of apoptosis-related cytokines mRNA: TNF-a (A), IL-6 (B), IL-1b (C) and IL-1a (D): qRT-PCR result was plotted and expressed as ΔCT that represent the difference between the gene of interest and the house keeping gene (b-actin) (*p<0.05).

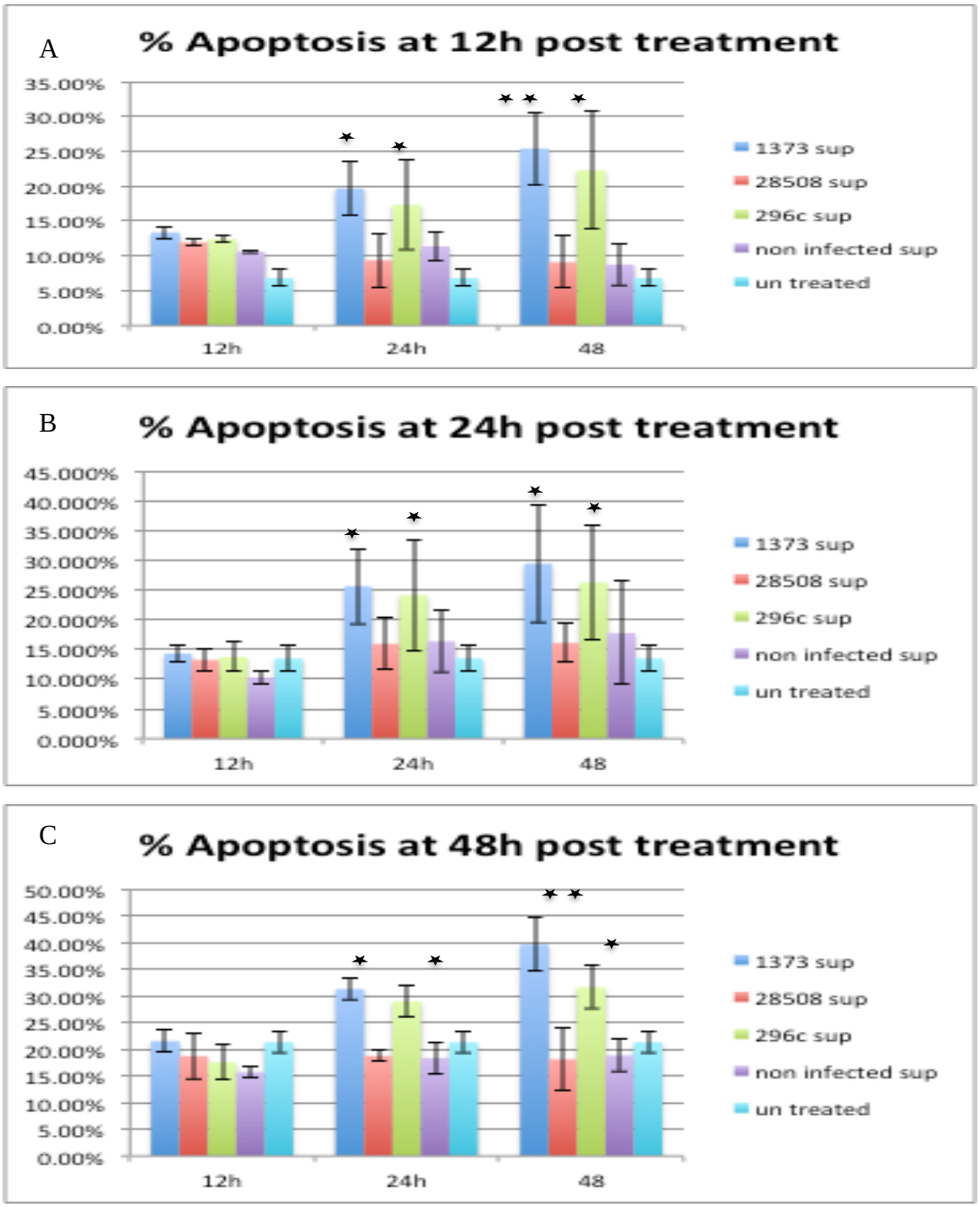

Fig 2.6. The indirect apoptotic effect of BVDV strains on peripheral blood total lymphocytes: at 12 (A), 24 (B) and 48 (C) h post treatment by 3 different time-point supernatants; 12, 24 and 48h sup of 3 different strains; 1373, 28508 and 296 C inn addition to the mock infected MDM sup and non-treated lymphocytes. (* $p<0.05$, ** $p< 0.01$)

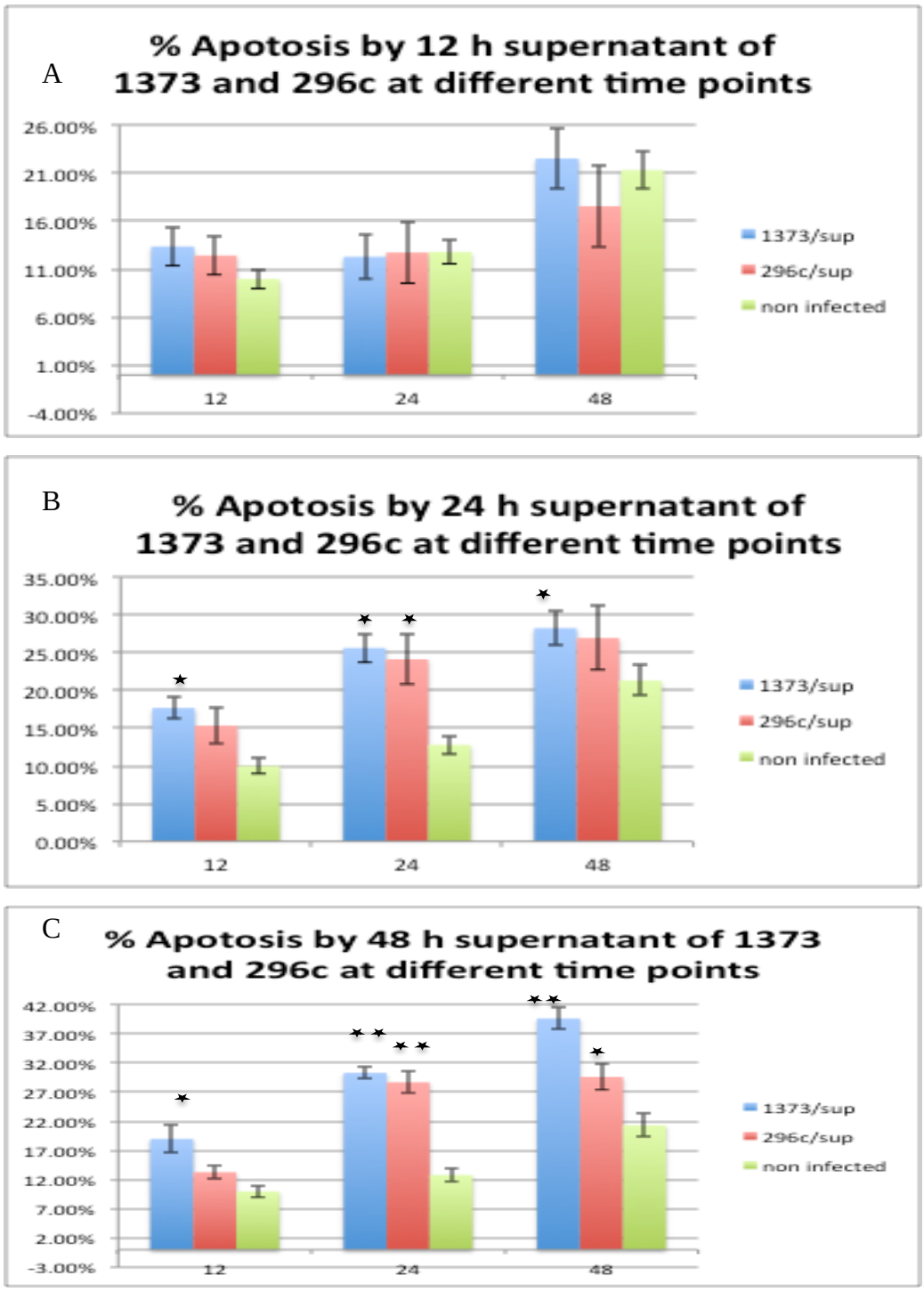

Fig 2.7. The indirect apoptotic effect of BVDV strains on BL-3 cells: at 12, 24 and 48 h post treatment by 3 different time-point supernatants; 12 (A), 24 (B) and 48 h(C) sup of 2 different strains; 1373 and 296 C inn addition to the mock infected MDM sup. (* $p<0.05$, ** $p< 0.01$)

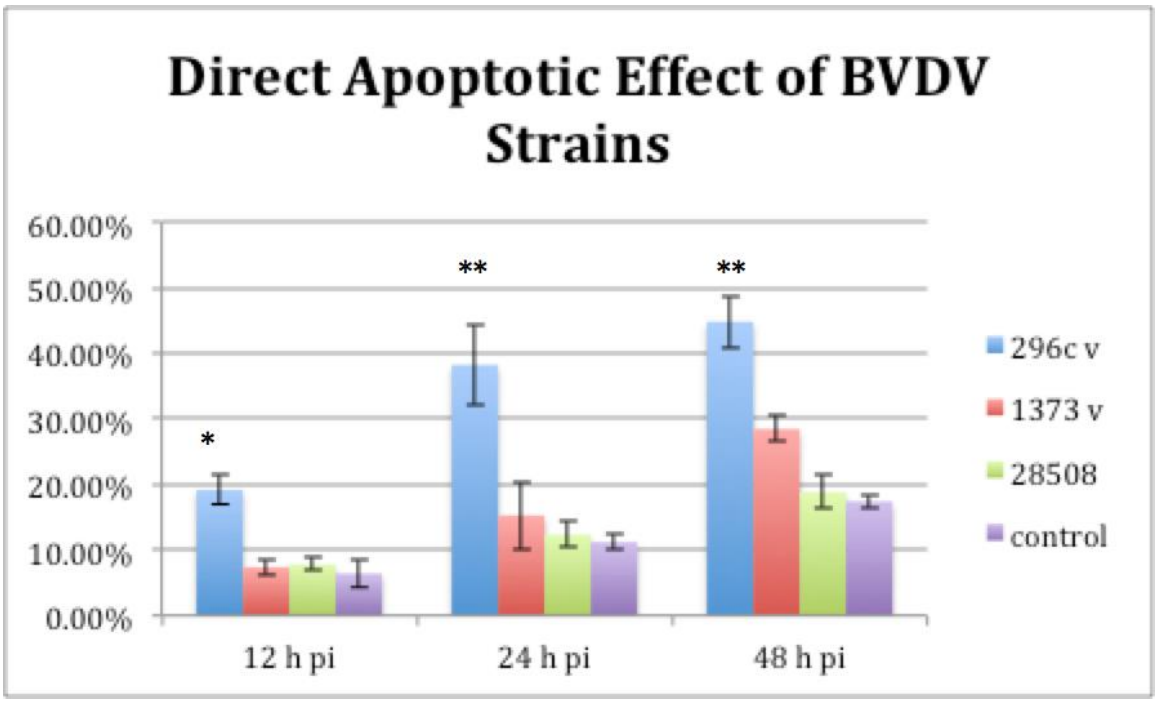

Fig 2.8. Direct apoptotic effect of BVDV strains on BL-3 cells: BL-3 infected with both CP 296 C, NCP 1373 and 28508 at 12, 24 and 48 h pi compared to mock-infected cells (*$p<0.05$, **$p<0.01$).

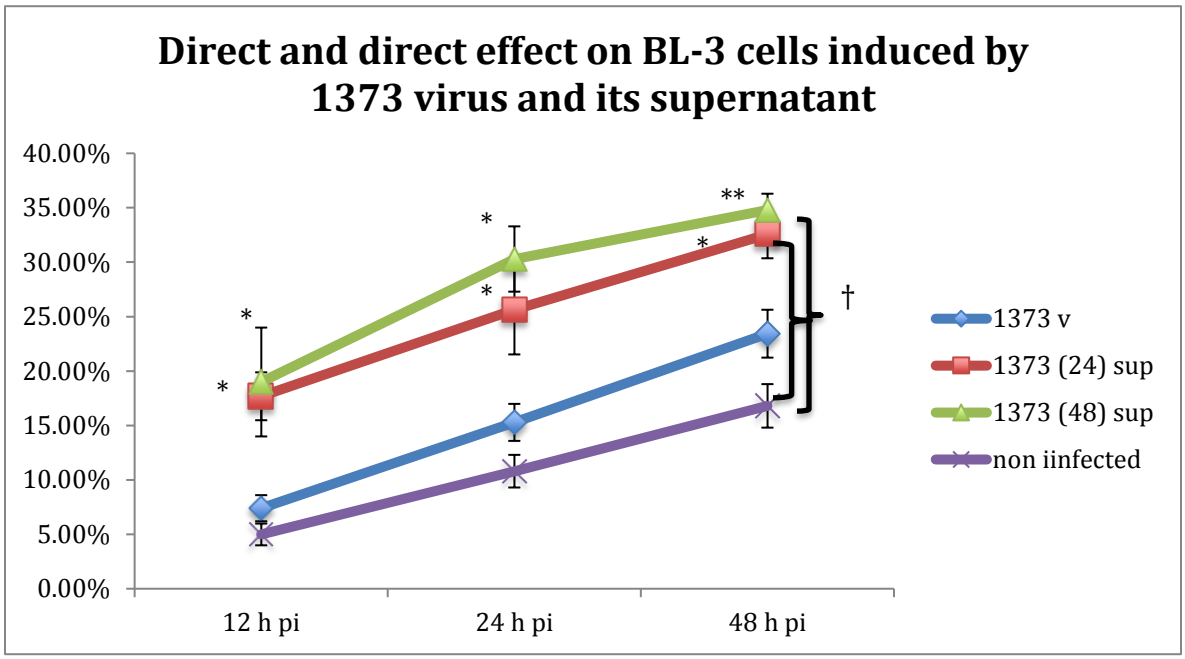

Fig 2.9. Direct and indirect apoptotic effect on BL-3 cells induced by 1373 virus and its supernatant: BL-3 cells were treated or infected by 2 time-points supernatants; 24 and 48h sup or 1373 strain and this was compared to mock-infected cells (*p<0.05, **p<0.01). † Significance was determined in contrast to non-infected control.

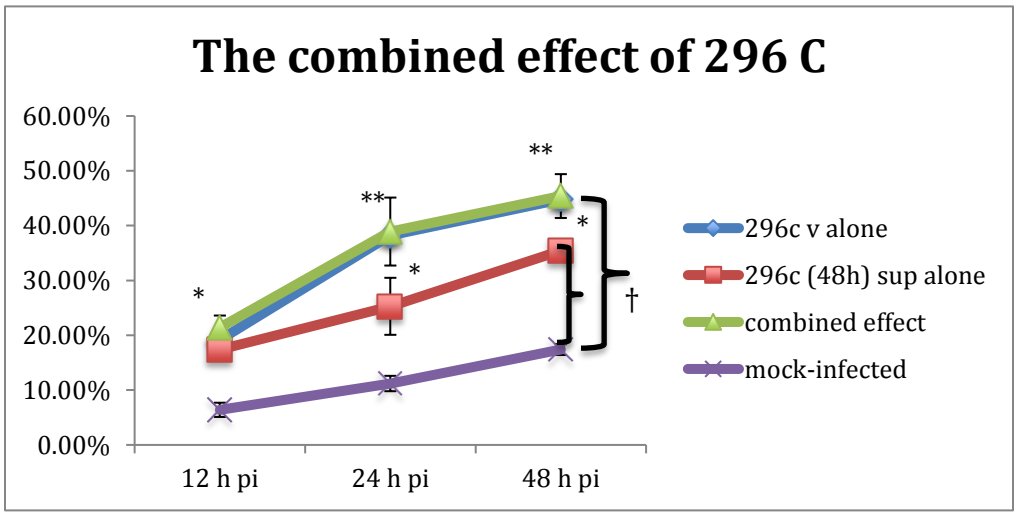

Fig 2.10. The combined effect of 296 C strains: the combined effect: (both direct and indirect), was compared to direct and indirect, each alone as well as the mock infected control. † Significance was determined in contrast to non-infected control. (*$p<0.05$, **$p<0.01$)

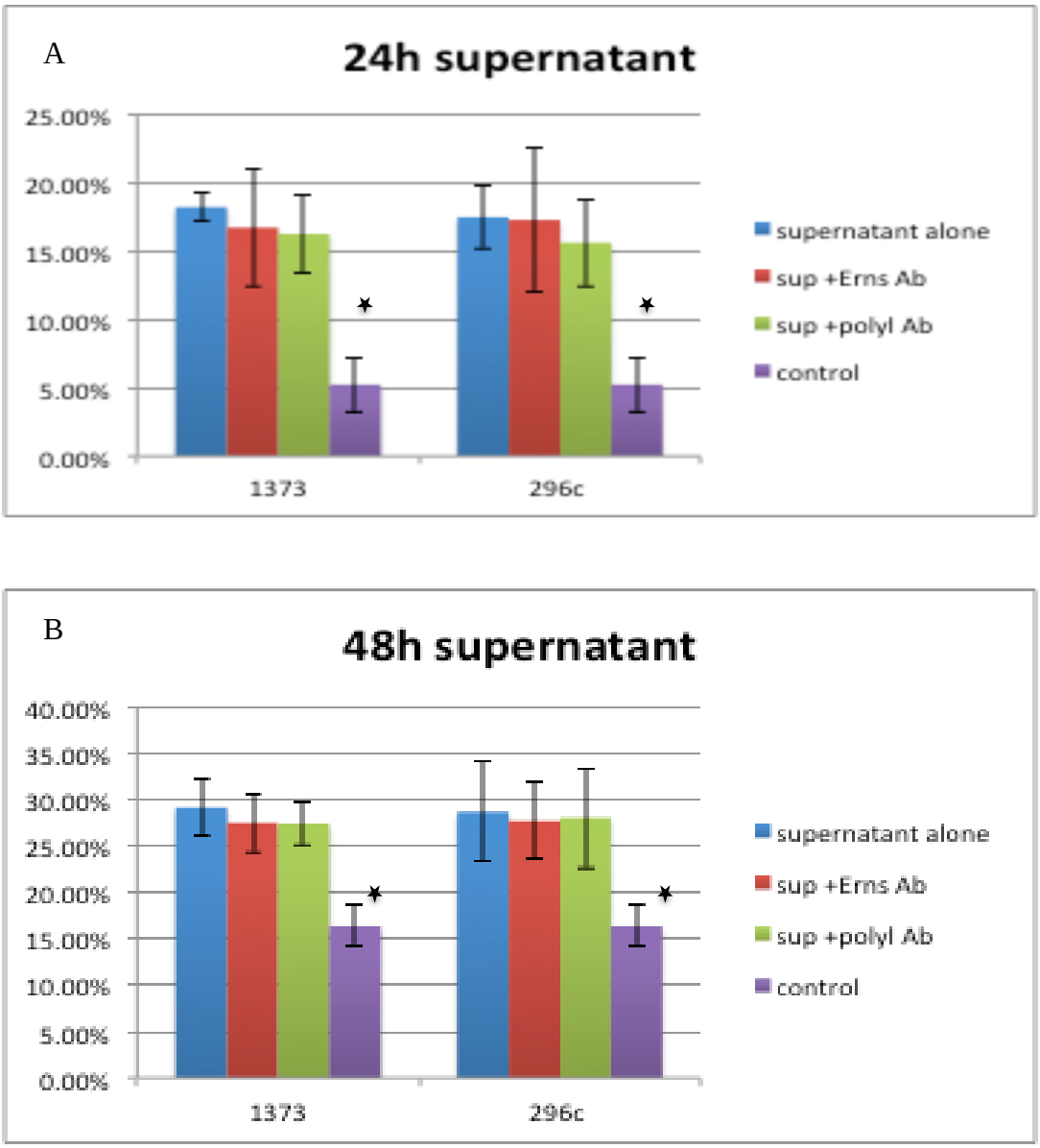

Fig 2.11. The role of viral proteins on BL-3 lymphocyte indirect apoptosis: 2 different time-point supernatants; 24 (A) and 48 (B) sup of infected MDM with 2 diff strains of BVDV; 1373 and 296 C were neutralized using 2 different antibodies; 15c5 E^{rns} specific monoclonal antibody and BVDV specific polyclonal antibody. This is in comparison to positive sup and mock infected BL-3 cells. This result obtained 36 h post treatment (*$p<0.05$)

BVDV strains	Biotype and Genotype	Virulence	References
1373	NCP BVDV-2	High virulence	Stoffregen et. al., 2000
28508	NCP BVDV-2	Low virulence	Carman et. al., 1998
296C	CP BVDV-2	Low virulence	Ridpath et al., 2006

Table 2.1. Different strains of BVDV used in the current study

	Cytokine	Forward Primer 5'- 3'	Reverse Primer 5'- 3'	Annealing Temp ^{o}C
1	TNF-a	AGA CCC CAG CAC CCA GGA CTC G	GGA GAT GCC ATC TGT GTG AGT G	55
2	IL-1a	GAT GCC TGA GAC ACC CAA	GAA AGT CAG TGA TCG AGG G	53
3	IL-1b	CAA GGA GAG GAA AGA GAC A	TGA GAA GTG CTG ATG TAC CA	53
4	IL- 6	TCC AGA ACG AGT ATG AGG	CAT CCG AAT AGC TCT CAG	52
5	b-actin	CGC ACC ACT GGC ATT GTC AT	TCC AAG GCG ACG TAG CAG AG	60

Table 2.2. The set of primers used for quantifications of apoptosis-related cytokine mRNA